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PATHOLOGICAL PHYSIOLOGY OF SALMONELLOSIS

II. PLASMA CHOLINESTERASE IN INFECTIONS DUE TO ENTEROBACTERIACEAE

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Cholinesterase activity in toxemias of diverse etiology has been studied from time to time with a uniform result of a moderate reduction in all such cases;[1–3] however, none of these works included an adequate number of patients with the same etiology. Recently Lach Zajacowa[4] studied blood cholinesterase in a great variety of infectious diseases (typhoid fever among them), and reported depressed enzymatic activity, more marked in severe cases, but, unfortunately, its values were not followed serially in each patient.

Aside from a pathologic evidence of liver damage in Enterobacteriaceae infections—especially in typhoid fever—there are studies with suggestive data to support the idea of a hepatic injury in gastroenteritis and diarrheas in general,[5,6] and Kun et al[7–9] have shown that endotoxins produced by Gram-negative bacteria interfere with the carbohydrate metabolism of the liver, thus inhibiting glycogen synthesis. The lack of a sensitive and quantitative test had obstructed the correlation of a real hepatic function and clinical evolution, but at present Michel's technique for plasma cholinesterase[10] has partly overcome that difficulty.

On this basis, the alterations in the normal values may be explained in three different ways: (a) direct inhibition by endotoxins or derived products, (b) modification of plasma volume in the presence of normal quantities of cholinesterase, and (c) alteration of the hepatic synthesis of the enzyme.

Plasma cholinesterase is described as a true guide of the nutritional state, but since in our children infections and malnutrition are frequently associated, the appraisal of the findings should be made in such a way as to eliminate corporal weight, one of the most repre-

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* With the assistance of Delfina Arrieta A.

1. Hall, G. E. and Lucas, C. C. 1937, Cholinesterase activity of normal and pathological sera. J. Pharmacol. **59:** 34–42.
2. Milhorat, A. T. 1938, The cholinesterase activity of the blood serum in disease. J. Clin. Investigation, **17:** 649–657.
3. Dikshit, B. B. and Mahal, H. S. 1938, Cholinesterase in toxaemia. J. Exper. Physiol. **27:** 41–48.
4. Lach-Zajacowa, M. 1954, Blood cholinesterase and infectious diseases. Med. Doswiadczalna i Mikrobiol. **6:** 299–312. Quoted from Chem. Abs. **49**/2: 490–h (Jan. 10, 1955).
5. Schlesinger, B., Payne, W. W. and Burnard, E. D. 1949, Liver damage in gastroenteritis. Arch. Dis. Childhood, **24:** 15–28.
6. Forsander, O. and Hallman, N. 1952. On free plasma choline in severe infantile gastroenteritis. Scand. J. Clin. Lab. Invest. **4:** 63–67.
7. Kun, E. and Miller, C. P. 1948, Effect of bacterial endotoxins and carbohydrate metabolism in rabbits. Proc. Soc. Exper. Biol. & Med. **67:** 220–225.
8. Kun, E. 1948, Effect of bacterial endotoxins on glycogen synthesis. Proc. Soc. Exper. Biol. & Med. **68:** 496–498.
9. Kun, E. and Abood, L. G. 1949, Mechanism of inhibition of glycogen synthesis by endotoxins of *Salmonella aertrycke* and type I meningococcus. Proc. Soc. Exper. Biol. & Med. **71:** 362–364.
10. Michel, H. O. 1949, An electrometric method for the determination of red blood cell and plasma cholinesterase activity. J. Lab. & Clin. Med. **34:** 1564–1568.

sentative elements of the nutritional state, as a contributing factor.

All data of plasma cholinesterase have been reported in relative terms without taking into account the total activity present in the plasma volume; this unknown volume of distribution makes it impossible to state, with a basis on the increase of the relative activity, if the circulating enzyme has changed in the same sense. In order to judge the evolution of the relative activity in relation to the total quantity of enzyme present, it became necessary in some cases to estimate plasma volume.

Considering these points, we tried to study the enzyme activity of plasma cholinesterase in children infected with pathogenic Enterobacteriaceae, following it serially to see its evolution along the course of the disease; these results were then correlated with the clinical response and compared with those obtained in children with diarrhea without detectable pathogenic agent.

MATERIAL AND METHODS

Thirty-three children infected with Enterobacteriaceae, confirmed in every case by blood and/or stool cultures, were divided as follows: 18 with *Salmonella typhi* (16 in blood and 2 only in feces), 9 with *Shigella flexneri* (only in feces), 4 with *Escherichia coli* 0111 (Escherichia coligomez) only in feces, and 4 with various salmonellas (*S. derby, S. paratyphi A, S. typhimurium* and an unidentified salmonella from group C) isolated in feces and in the second in blood culture.

In 2 of these cases there was association of 2 bacteria: *S. derby* and *Sh. flexneri*, and *S. typhimurium* and *Esch. coli* 0111.

With the exception of 3 cases, all recovered; 2 died because of toxemia, and the third became complicated by post-operative sequelae due to perforation from typhoid fever.

One hundred normal children previously studied[11] served as control to judge the recovery of normal values. Eighteen children with diagnosis of "infectious enteral diarrhea," in whom no pathogenic germ was found in at least 3 stool and blood cultures at daily intervals, served as control group to elucidate the effects derived from pathogenic bacteria.

Plasma cholinesterase was determined by Michel's technique[10] and its activity was expressed as ΔpH/hour; this unit is calculated by use of Michel's formula:

$$\text{pH/hour} = \left(\frac{\text{pH}_1 - \text{pH}_2}{t_2 - t_1} - b \right) f$$

where pH_1 = initial pH, pH_2 = final pH, t_1 = time of mixing with acetylcholine, t_2 = time of reading pH_2, b = nonenzymatic hydrolysis correction corresponding to pH_2, f = correction for variations in ΔpH/hour with pH, corresponding to pH_2. The corrections for b and f vary between 0.01–0.09 in b and between 0.98–1.09 for f as given by Michel.

Bacterial endotoxins were obtained by Walker's technique[12] and plasma volume, by means of Evans blue technique, as described by Gibson and Evans adapted for Coleman Junior spectrophotometer.[13]

Statistical analysis was conducted in the manner described in standard treatises on the subject.[14]

The weight was registered at least weekly, coinciding with the determination of enzyme activity. The plasma volume was measured in 9 cases of typhoid fever in whom 24 simultaneous determinations of plasma cholinesterase were made.

The corporal area was determined by means of Boothby and Sandiford's monogram[15] derived from DuBois' formula.

Typhoid fever and salmonella cases were treated with chloramphenicol, synnematin B, or tetracycline; 4 children with shigella were given sulfadiazine at the rate of 200 mg/kg/day for periods of 4 to 5 days. The control group was

11. Benavides V., L., Kumate, J., Criollos, O., Carrillo, J. and Pérez Navarrete, J. L. 1954, Colinesterasa plasmática y acetilcolinesterasa globular en niños normales. Bol. med. Hosp. infant. Mex. **11**: 265–277.
12. Walker, J. 1940, A method for the isolation of toxic and immunizing fractions from bacteria of the Salmonella group, Biochem. J. **34**: 325–329.
13. Gibson, J. G., II and Evans, W. A., Jr. 1937, Clinical studies of the blood volume; clinical application of a method employing the azo dye "Evans blue" and the spectrophotometer. J. Clin. Investigation, **16**: 301–316.
14. Croxton, F. E. 1953, Elementary Statistics with Applications in Medicine, New York, Prentice-Hall, Inc.
15. Crawford, J. D., Terry, M. E. and Rourke, M. 1950, Simplification of drug dosage calculation by application of the surface area principle. Pediatrics, **5**: 783–789.

managed symptomatically by means of diet and parenteral fluids; in 6 patients, sulfadiazine was given at the same doses as those employed in the shigella group.

RESULTS

In the 33 cases, 122 serial determinations were made, from the 8th to the 46th day of the disease. The range of values of enzyme activity was between 0.27 and 1.70 ΔpH/hour.

The 30 cured or recovered cases were plotted in a scatter diagram (fig. 1). The regression equation was

$$y = 0.01736x + 0.31;\ r = 0.6345,$$
$$S_{yx} = 0.21 \text{ and } t = 7.46,$$

with $P < 0.001$.

Their grouping from the second to the 5th weeks is shown by a series of levels which go from 0.495 to 0.93 ΔpH/hour, passing through 0.61 and 0.72 in the 3rd and 4th weeks. Standard deviations were 0.15, 0.18, 0.18, and 0.25, respectively, with N of 34, 28, 24 and 21 cases. Statistically significant differences were found at each level, both with that immediately below, as with that above (fig. 2). The weekly grouping of cases with salmonellosis, shigellosis and colibacillosis, respectively, showed the same tendency.

The weight increase factor and course of enzyme activity were correlated in both groups. In the salmonella group, the deficiencies in initial weight, which were not greater than 35%, were grouped around 20% (calculated in relation to the ideal weight for the age); $r = 0.1467$; $t = 1.039$, which does not give significance to $P > 0.25$ (fig. 3). In

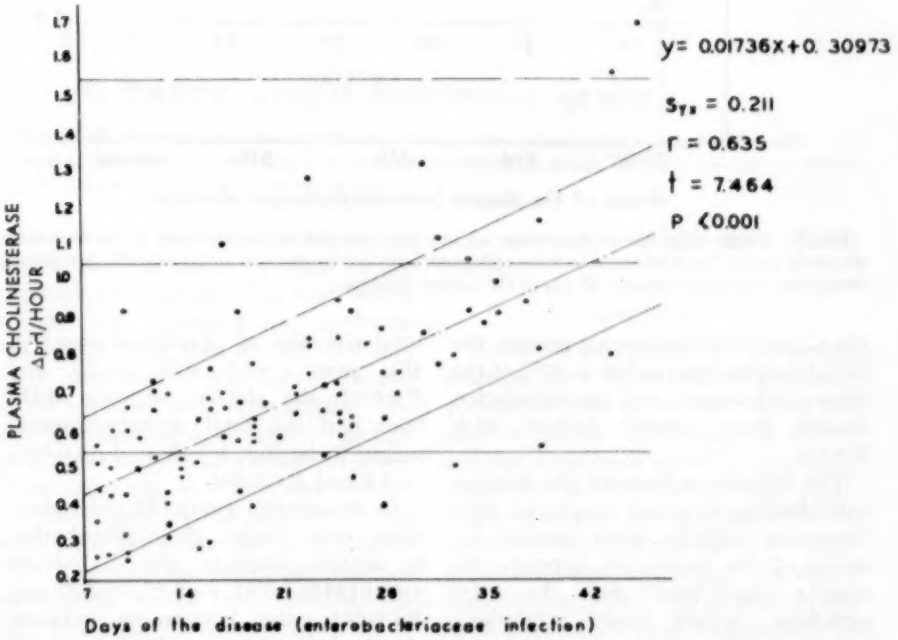

FIG. 1.—Evolution of plasma cholinesterase activity in uneventful Enterobacteriaceae infections. The group comprises 30 proved (blood and/or feces cultures) cases with 99 serial determinations. Horizontal dotted line represents the mean of enzyme values in 100 normal children; the limits M $\pm 2\sigma$ are denoted by full lines.

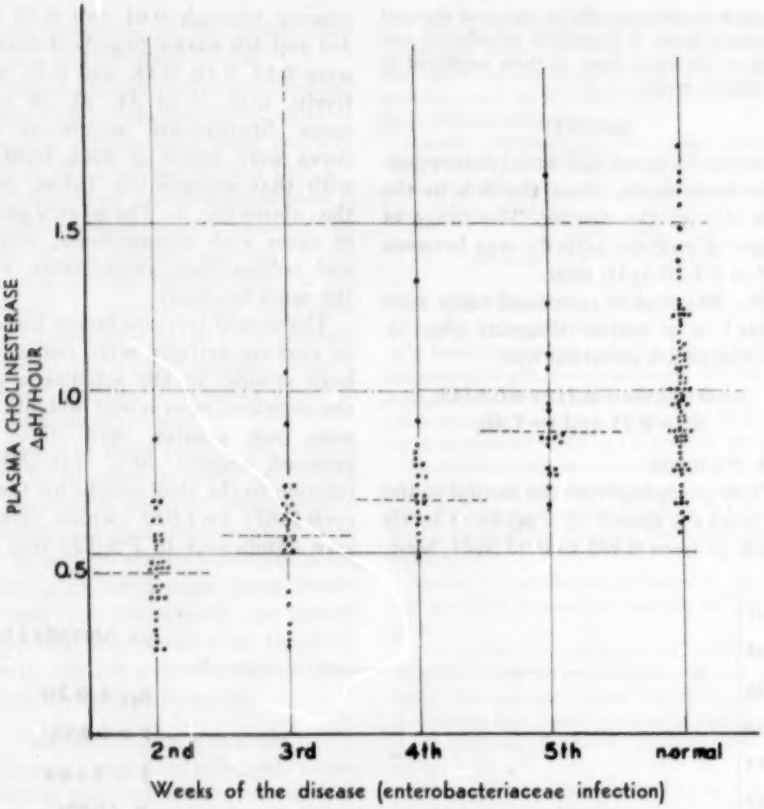

FIG. 2.—Levels of plasma cholinesterase activity from the 2nd to the 5th week of the disease in the whole group. The differences between each mean level and its next one are statistically significant. Dotted and full lines represent $M \pm 2\sigma$ of 100 normal children.

the shigella and escherichia groups, the initial weights were as low as 40% of the theoretical weight, and the correlation showed that $r = 0.088$, $t = 0.46$, with $P > 0.1$.

The differences between the relative and absolute increases in plasma cholinesterase activity were studied by means of the correlation between the relative (ΔpH/hour) and the total activities [(ΔpH hour) (dilution) (plasma cholinesterase space = plasma volume in liters)] in figure 4a: $r = 0.59$; $t = 3.4$, which gives $P < 0.001$ for $N = 24$. Correlation between the relative and the total activities/m^2 of corporal area (fig. 4b), gave $r = 0.5$ with $t = 2.7$ and $P < 0.02$; the plotting between ΔpH/hour and the total activity/corporal weight in kg (fig. 4c) showed $r = 0.675$, $t = 4.2$ and $P < 0.001$.

In the control group, 52 determinations were made; their distribution in scatter diagram (fig. 5) shows $y = 0.01842x + 0.61$; $r = 0.45$; $t = 3.57$ and $P < 0.001$; the correlation between plasma cholinesterase and weight in this group was erratic and without clear tendency (fig. 6).

In the elim nated cases there was

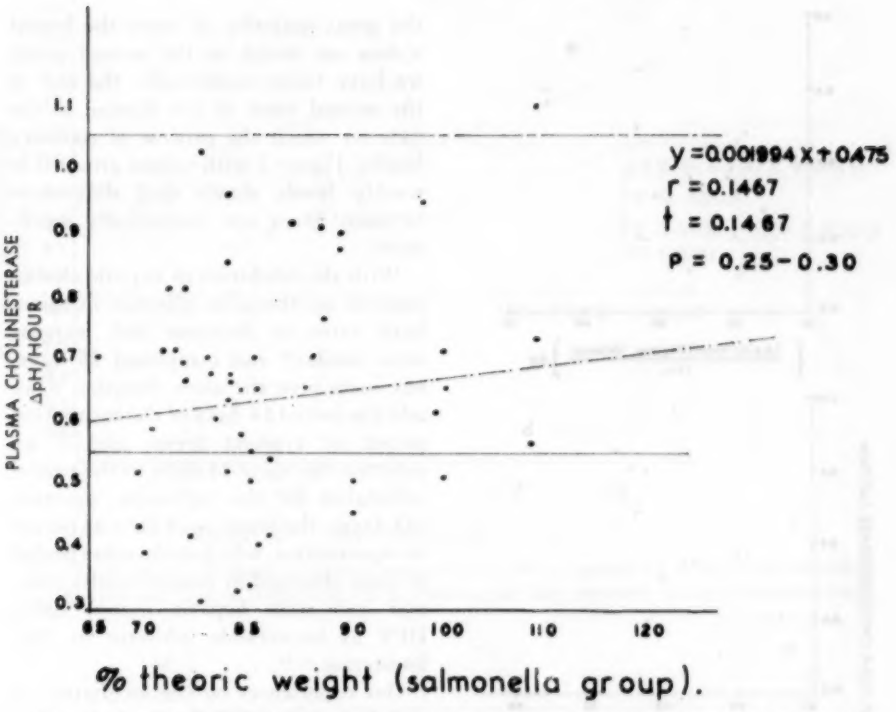

FIG. 3.—Scatter diagram between plasma cholinesterase activity (ΔpH/hour) and weight (expressed as percent of theoretic weight for their age) in salmonella group. Dotted line = mean of normal values; full line = mean -2σ of normal values.

association of abnormal circumstances which preclude their inclusion in the typhoid fever group. The first was a severe case, resistant to antibiotic treatment, who died on the 6th day after admission (19th day of illness) from toxemia and myocarditis. The second associated to the typhoid picture and hepatic coma as a result of infectious hepatitis, and although she recovered from the coma, she died 3 days later with peritonitis due to a perforation. The last case presented intestinal perforation which was treated opportunely, but his postoperative condition was complicated by hernias which required 4 more operations. In every case, enzyme values, low in themselves, showed tendencies to decline even more, and reflected faithfully the clinical course.

DISCUSSION

Statistical analysis of the first scatter diagram (fig. 1) shows clearly a positive correlation between enzyme values and days of disease (uneventful); if the regression equation is solved for $y = 1.0518$ (mean for the 100 normal cases previously studied), $x = 42.7$ days, which would be the time necessary for complete recovery to normal values of cholinesterase. In the present study there were only 3 cases with determinations previous to the 8th day serially followed, in whom, after a drop to lower values in the second week, a rise to normal values was observed. Since in

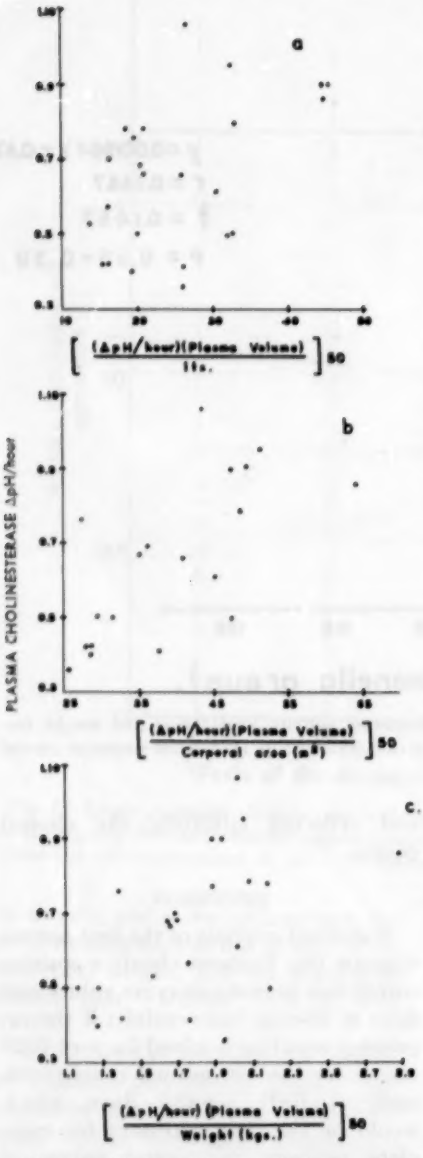
a
b
c.
PLASMA CHOLINESTERASE ΔpH/hour
[(ΔpH/hour)(Plasma Volume) / Lts.] 50
[(ΔpH/hour)(Plasma Volume) / Corporal area (m²)] 50
[(ΔpH/hour)(Plasma Volume) / Weight (kgs.)] 50

FIG. 4.—Scatter diagrams between plasma cholinesterase activity (ΔpH/hour) and its distribution volume in typhoid fever cases, expressed as:

a (ΔpH/hour) (plasma volume in liters) 50*

b $\frac{(\Delta pH/hour)(plasma\ volume\ in\ liters)\ 50^*}{corporal\ area\ in\ square\ meters}$

c $\frac{(\Delta pH/hour)(plasma\ volume\ in\ liters)50^*}{corporal\ weight\ in\ kgs}$

the great majority of cases the lowest values are found on the second week, we have taken statistically the end of the second week of the disease as the date on which the process of recovery begins. Figure 2 with values grouped in weekly levels shows that differences between them are statistically significant.

With the inhibition of hepatic cholinesterase synthesis by albumin infusion, both rates of decrease and increase were similar[16] and comprised 28 days; our cases give the same duration if we add the initial 14 days of the incubation period of typhoid fever; and if we subtract the same 14 days to the period calculated for the regression equation (42 days), the result is 28 days as period of regeneration, which is the same period of time obtained in human adults (normal and with hepatic lesion) using DPF as irreversible inhibitor of cholinesterase.[17,18]

Our experiences on the incubation of endotoxins from salmonella and pathogenic escherichia with human serums—as source of cholinesterase—do not reveal any inhibitory effect on the enzyme activity; the range of concentrations used varied from 50 to 100 μg of endotoxin (obtained by Walker's technique)/ml of serum.

Correlation studies between relative and absolute activities show a significant association of a direct type, as much for total activity, as m^2 of cor-

* Dilution factor in Michel's technique for plasma cholinesterase.

16. Vorhaus, L. J. and Kark, R. M. 1953, Serum cholinesterase in health and disease. Am. J. Med. **14**: 707–719.

17. Vorhaus, L. J., Scudamore, H. H. and Kark, R. M. 1950, Measurement of serum cholinesterase activity: a useful tool in the liver and biliary system. Gastroenterology, **15**: 304–315.

18. Grob, O., Lilienthal, J. L., Harvey, A. M. and Jones, B. F., 1947, The administration of di-isopropyl fluorophosphate (DPF) to man. Bull. Johns Hopkins Hosp. **81**: 217–244.

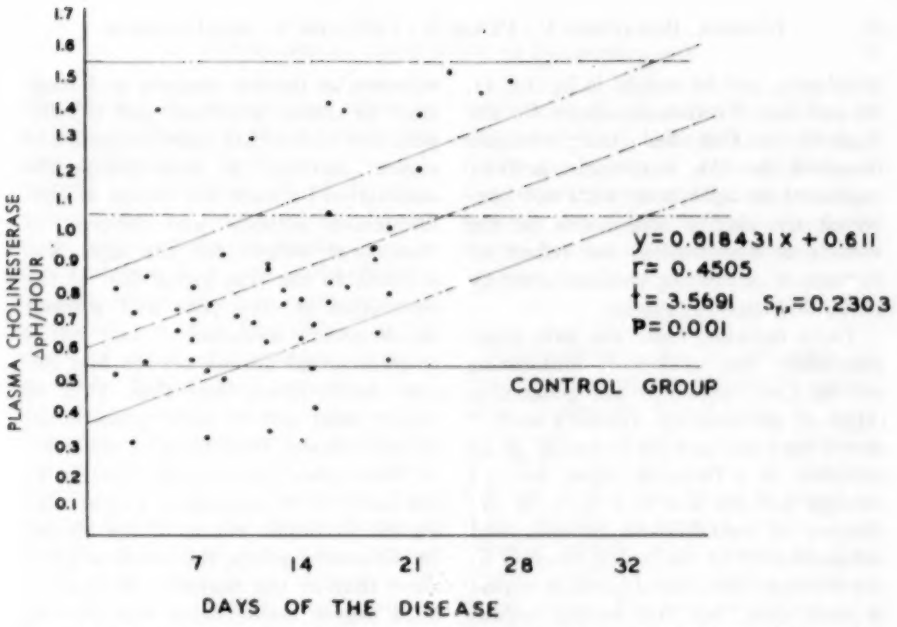

FIG. 5.—Evolution of plasma cholinesterase activity in control group—18 children with noninfectious diarrhea and 52 serial determinations. Dotted and full lines represent $M \pm 2\sigma$ of normal values.

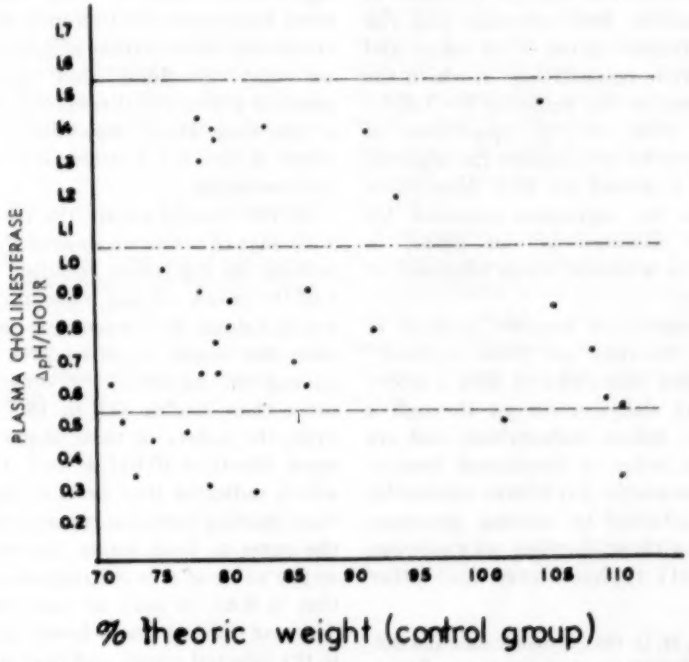

FIG. 6.—Scatter diagram between plasma cholinesterase activity (ΔpH/hour) and weight (expressed as percent of theoretic weight for their age) in control group. Dotted and full lines represent $M \pm 2\sigma$ of normal values in children.

poral area, and for weight in kg (fig. 4a, 4b and 4c). We then conclude, for the typhoid cases studied, that increases observed in the enzymatic activity expressed as ΔpH/hour were not contrived by inverse alterations in the volume of distribution, but reflect an increase of circulating cholinesterase by probable hepatic synthesis.

There remains, then, the only other possibility, the action of endotoxins on the liver, which is the generating organ of the enzyme. Levine's study[19] shows that the enzyme is found, as all proteins, in a dynamic state, and its average half-life is 6 to 7 days; in the absence of extrahepatic factors, and independently of the value reached in the decrease, the rate of increase varied in each case, but the normal values (before illness) were reached in a period of 28 days. If the fall is considered a result of disturbed hepatic synthesis of cholinesterase, both decrease and rise may be treated as curves of decay and regeneration, respectively, in which the application of the equation $k = 2.303/t \log c^0/c$ yield $c = 5\%$ (quantitity of enzyme needed to complete the original) when it is solved for 42.7 days (time calculated by regression equation for complete recovery in our cases) in accordance with the value obtained in our study.

The reports of hepatic damage in cases of diarrhea are from authors[5,6] who suggest that children with a severe process of dehydration go through a period of serious malnutrition and are prone to suffer a functional hepatic lesion afterwards. As children studied by us were affected by toxemic processes with and without diarrhea, we made two groups: (1) typhoid fever and other salmonellas (severe toxemia and moderate or absent diarrhea) and (2) shigella and escherichia (mild toxemia and severe diarrhea). In both groups the association between the course of cholinesterase activity and percent of theoretical weight for the ages was studied. In the first group (fig. 3) the correlation is very poor and without significance; moreover, the initial weights reveal almost always first degree malnutrition (less than 15% of weight loss) and in some cases none; for that reason, the effects on the liver, in these cases, are not attributable to the factor of an increase in weight during the favorable course of the illness. In the second group, the initial weights show that in the majority of cases a third degree malnutrition was present (more than 40% of weight loss); the correlation is very poor and without significance. Since in those cases diarrheal hemoconcentration may equal extracellular fluid increase of malnutrition, we may conclude that endotoxins produce a functional reversible damage of the liver which shows itself, among other things, by a decrease of plasma cholinesterase.

In the control group, the correlation with days of evolution showed $P < 0.001$. Solving its regression equation for $y = 1.0518$ (mean of the normal values), $x = 18.5$ days. If this figure is compared with the result in cases with proved pathogenic bacteria, the difference is more than double (42 to 18.5). However, the values of their slopes are almost identical (0.01736 and 0.01843), which indicates that rates of regeneration, starting from the lowest level, were the same in both series. Nevertheless, as the value of a in the regression equation is 0.61, it may be seen that the decrease did not reach levels as low as in the infected group, and that an extrahepatic factor intervened to hasten the

19. Levine, M. G. 1953, Dynamic state and half-life of human serum cholinesterase. Experientia, 9: 101–103.

recuperation of the enzyme values in plasma. In this group, the initial weights were comparable to those in the salmonella group (above 75% of the theoretical weight for their ages) and did not show relation with the plasma level of cholinesterase. It is very likely that the changes found in these cases are due exclusively, in their first phase, to the effect of acute malnutrition, as suggested by Schlesinger,[5] and the reason for cholinesterase values higher than those found in salmonella and shigella /escherichia groups could be a milder hepatic lesion, or the hemoconcentration secondary to the acute diarrheal process, or both of them; the greater speed of recovery is not a process of pure recovery, but with our present data we can not offer an explanation.

As Zeller[20] has described inhibition of cholinesterase ranging from 10 to 30% by certain sulfonamides at therapeutic doses, our cases receiving sulfas were analyzed on the basis of a possible association of low enzymatic values during such therapeutic period; such relation was found only in 2 cases, but they were excluded from statistical studies. Although Smith et al[21] have described inhibition of bacterial esterase by chloramphenicol at high doses and slight inhibition of crystalline horse liver esterase over a wide concentration range, a rise of enzyme values occurred during therapy in our cases treated with antibiotics.

20. Zeller, E. A. 1940, Inhibition of cholinesterase by sulphonamides and diamines. Helv. Chim. Acta. **25**: 216–229.
21. Smith, G. N., Worrel, C. S. and Swanson, A. L. 1949, Inhibition of bacterial esterases by chloramphenicol (chloromycetin). J. Bact. **58**: 803–809.

SUMMARY

Thirty-three cases of infection by Enterobacteriaceae, showing pathogenic forms in blood and/or feces (*Salmonella*, *Shigella* and *Escherichia coli* 0111), and 18 cases of diarrhea without pathogenic forms in blood or feces, serving as control group, were followed serially with regard to their clinical condition and plasma cholinesterase values. The enzyme activity was consistently found at low levels in the early stages with a tendency to increase as the clinical condition improved. Contrastingly, in those cases ending fatally, or having a slow recovery, the enzyme activity showed a further decrease, or remained stationary at significantly low levels. The period of recovery was calculated at 28 days for the infected group, and 18.5 days for the control.

The recovery of the enzyme activity to normal levels is thought to be due to liver synthesis with no relation to increase in weight, to alterations in volume distribution, or to direct action of endotoxins on the circulating enzyme.

EXPERIMENTAL RABIES INFECTION IN INSECTIVOROUS BATS

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The study of past outbreaks of rabies in cattle and humans in South and Central America has shown that the vampire and the fruit-eating bats of those areas at times may disseminate the virus[1–5]. These bats were shown to be susceptible to experimental rabies infection, from which there were occasional survivors.[6,7] Later studies have demonstrated that the insectivorous bats of North America also may be infected experimentally,[8,9] and recent reports of the finding of naturally infected insectivorous bats in the United States[10–12] have raised the question of the possible role of bats in the ecology of rabies in this country. For this reason studies were initiated on the maintenance of insectivorous bats under laboratory conditions, on their reaction to experimental rabies infection, and on variations in infections produced by preparations of rabies virus isolated from several different sources.

MATERIALS AND METHODS

Three species of bats were employed in the studies. Specimens of *Myotis lucifugus* and *Pipistrellus subflavus* were obtained from Dr. Ernest P. Edwards, Frederick, Maryland. These two species were collected during hibernation in caves in western Maryland, shipped under refrigeration, and stored in a refrigerator for several days before inoculation. These bats became fully active within 10 minutes after being removed from the refrigerator. Specimens of *Eptesicus fuscus* were obtained from James B. Cope of Richmond, Indiana, and from Bryan P. Glass of Stillwater, Oklahoma. These bats arrived in fully active condition. All of the bats under observation were kept in 8 inch × 10 inch × 5 inch metal boxes with wire mesh tops. Their diet consisted of 6 to 8 chopped crickets per day and water, which they took readily from petri dishes.

Four different preparations of rabies street virus were employed. The first preparation, "Alaska wolf M_2IL_1," originally isolated from a wolf brain, had had two intracerebral and one intralingual mouse passages in the laboratory. The second preparation, "Florida bat #250", consisted of a 5% suspension of brain material from a naturally infected bat collected in Florida. The third preparation employed, "Florida bat #61 M^1," consisted of 10% mouse brain material representing the first intracerebral mouse passage of brain material from a naturally infected bat. The fourth preparation, "Florida bat #682 M_3IL_1," consisted of 10% mouse brain infected with a strain of rabies virus isolated from a naturally infected bat. This strain had undergone 3 intracerebral and 1 intralingual mouse passages.

As each of the preparations was inoculated into bats it was titrated by the intracerebral inoculation of tenfold dilutions into 3- to 4-week-old CFW mice. The total dose inoculated is expressed as mouse intracerebral LD_{50}'s.

Intracerebral inoculations of both mice and

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1. Haupt, H. and Rehaag, H. 1921, Ztschr. f. Infektionskr. **22**: 104–127.
2. Hurst, E. W. and Pawan, J. L. 1932, J. Path. & Bact. **35**: 301–321.
3. Pawan, J. L. 1936, Ann. Trop. Med. & Parasitol. **30**: 101–130.
4. Johnson, H. N. 1948, Am. J. Hyg. **47**: 189–204.
5. Malaga-Alba, A. L. 1953, Texas Health Bull. **6**: 4–7.
6. Pawan, J. L. 1936, Ann. Trop. Med. & Parasitol. **30**: 401–42.
7. Pawan, J. L. 1948, Ann. Trop. Med. & Parasitol. **42**: 173–177.
8. Reagan, R. L. and Brueckner, A. L. 1951, Cornell Vet. **41**: 295–298.
9. Reagan, R. L., Delaha, E. C. and Brueckner, A. L. 1954, Cornell Vet. **44**: 318–321.
10. Venter, H. D., Hoffert, W. R., Scatterday, J. E. and Hardy, A. V. 1954, Am. J. Pub. Health, **44**: 182–185.
11. Witte, E. J. 1954, Am. J. Pub. Health **44**: 186–187.
12. Burns, K. F., Farinacci, C. J. 1954, Science **120** (no. 3118): 548.

bats were made with a 27-gauge ½ inch needle and ½ ml syringe, and in an identical manner except that a volume of 0.02 ml was inoculated into the bats and a volume of 0.03 ml was inoculated into the mice. Intramuscular inoculations of bats were made with the same size syringe and needle. Injections were made into the heavy muscle over the breast using a volume of 0.05 or 0.1 ml.

Collections of saliva were made by swabbing the mouth with a small pledget of cotton held in thumb forceps. The cotton was soaked in ½ ml of horse serum in a flat bottom vial and the excess pressed out against the sides of the vial. Bats, when touched on the back, open their mouths to complain, allowing the introduction of the swab. After about 10 seconds of swabbing, the pledget of cotton was thoroughly agitated in the horse serum. The process was repeated three times for each collection. To control bacterial contamination, 500 units of penicillin and 1.0 mg of streptomycin were added to each ½ ml sample. The mixture was then allowed to incubate for ½ hour at room temperature before being inoculated intracerebrally into mice. Rabies virus was considered isolated when Negri bodies could be demonstrated in brain smears from mice which died following a typical course of symptoms. Five mice were inoculated with each specimen and observed for a 30-day period before being discarded as negative.

When deaths of bats occurred the brain and salivary glands were removed. A smear of the brain was made and stained with Sellers' stain for detection of Negri bodies. The remainder of the brain was then ground in a mortar with 2.0 ml of normal horse serum plus antibiotics (1000 units of penicillin and 2.0 mg streptomycin per ml), centrifuged, and the supernatant fluid inoculated intracerebrally into mice to determine the presence of rabies virus. The salivary glands were prepared for inoculation in a manner similar to that of the brains except that a smaller volume (1.0 ml) of normal horse serum was used.

RESULTS

Four specimens (no. 1, 2, 3, 4) of *M. lucifugus* were inoculated intracerebrally with 14,000 mouse intracerebral LD_{50}'s of the the Alaska wolf preparation of virus. Deaths occurred on the 10th, 17th, 20th and 21st days after inoculation. Negri bodies were present in the brains of three of these bats. Rabies virus was isolated from the brains of all four bats and from the salivary glands of one.

Three specimens (no. 5, 6, 7) of *M. lucifugus* were inoculated intramuscularly with 35,000 mouse intracerebral LD_{50}'s of the Alaska wolf preparation of virus. Members of this group died on the 20th, 27th, and 33rd days. The brain of one was Negri-positive; two were Negri-negative. Virus was isolated from the brains of all three, but from the salivary glands of only one (no. 6). Saliva samples were collected from one of this group (no. 7) on the 28th, 32nd and 33rd days, but no virus was isolated from any of the samples.

The next group consisted of four specimens (no. 8, 9, 10, 11) of *M. lucifugus* which were inoculated intracerebrally with 0.29 mouse intracerebral LD_{50} of the Florida Bat #250 preparation of virus. One of this group (no. 8) became aggressive on the 24th day after inoculation and was killed on the 25th day. Virus was isolated from the brain although the brain was Negri-negative and no virus was isolated from the salivary glands. A member of this group died on the 39th day and another on the 61st day. The brains of both were Negri-negative; no virus was isolated from the brain or salivary glands of either; therefore, these deaths were considered to be nonspecific. The fourth member of this group (no. 11) died on the 50th day after inoculation. The brain was Negri-positive and virus was isolated from both brain and salivary glands. The only symptom displayed was decreased appetite for the last 4 days before death. Saliva samples had been collected from this bat on the 32nd through the 50th days. Rabies virus was isolated from those samples collected on the 36th, 37th, 39th, 43rd, 46th, 48th, and 49th days.

A group of three (no. 12, 13, 14) *M. lucifugus* were inoculated intramuscu-

larly with 0.71 mouse intracerebral LD_{50} of the Florida Bat #250 preparation of virus. Deaths occurred on the 61st, 82nd, and 129th days after inoculation. No signs of disease were apparent before death; the brains of all three were Negri-negative; and no virus was isolated from the brains or salivary glands of any of the group. Saliva samples were collected from these bats daily beginning on the 32nd day after inoculation and continuing until death of the animal. No virus was isolated from any of these samples. Therefore, these deaths were considered to be nonspecific.

Two specimens (no. 15, 16) of *P. subflavus* were inoculated intramuscularly with 17 mouse intracerebral LD_{50}'s of the Florida Bat #61 preparation of virus. Both bats died on the 53rd day and no symptoms had been noted before death. Both brains were Negri-negative; no virus was isolated from the brains or salivary glands, nor from daily saliva samples. It is assumed that the deaths of these bats were due to causes other than rabies.

Two specimens (no. 17, 18) of *M. lucifugus* were inoculated intramuscularly with 17 mouse intracerebral LD_{50}'s of the Florida Bat #61 preparation of virus. These animals remained normal through the 126th day after inoculation. Samples of saliva were collected from each bat on the 3rd through the 80th days. No virus was isolated from any of these samples. These two bats were then inoculated with 13,300 mouse intracerebral LD_{50}'s of the Alaska Wolf preparation of virus. One specimen (no. 18) died on the 19th day after inoculation, having shown symptoms of irritability, circling, and biting at the site of inoculation. The brain of this bat was Negri-negative; virus was isolated from the brain but not from the salivary glands. The second specimen (no. 17) died on the 31st day after inoculation; no definite symptoms of disease were noted before death; the brain was Negri-positive; and virus was isolated from the brain but not from the salivary glands. No virus was isolated from saliva samples collected daily from each bat from the 3rd day until death.

Five specimens (no. 19 to 23) of *E. fuscus* were inoculated intramuscularly with 170 mouse intracerebral LD_{50}'s of the Florida Bat #682 preparation of virus. Four bats of this group died on the 31st, 38th, 43rd, and 49th days, all having shown symptoms compatible with furious rabies, including aggressiveness and extreme irritability, within 24 hours before death. The 5th bat of this group died on the 65th day after refusing to eat for 5 days and becoming progressively weaker. The brain of the bat (no. 19) which died on the 43rd day was Negri-positive; the brains of the other four were Negri-negative. No virus was isolated from the brain, salivary glands, or from daily saliva samples from any of these bats.

Information on the 12 bats which died of confirmed rabies infection is tabulated in table 1. Those bats dying from rabies showed symptoms lasting from 3 to 13 days. The symptoms consisted of loss of appetite, weakness, tremors, irritability, muscular spasms, paralysis, biting at the site of inoculation, and aggressiveness. Negri bodies could not be demonstrated in the brains of 5 of the 12 bats dying of rabies. In one case Negri bodies were present in the brain but virus could not be recovered. Three of these 12 bats had demonstrable virus in the salivary glands.

Attempts to establish a pattern of appearance and persistence of virus in the saliva failed except in the case of one bat. Two of the bats which showed virus in the salivary glands died before it was known how much handling they would tolerate; therefore collection of saliva was not attempted. The saliva of the third bat (no. 11), which had virus

TABLE 1.—*Experimental rabies in insectivorous bats.*

Bat no.	Species	Route of inoculation	Virus strain and dosage	Day of death after inoculation	Negri bodies in brain	Rabies virus in brain	Rabies virus in salivary gland	Days saliva collected and presence of virus
1	*M. lucifugus*	IC	Alaska wolf 14,000 mouse IC LD_{50}	10th	−	+	−	
2	*M. lucifugus*	IC	14,000 mouse IC LD_{50}	17th	+	+	−	
3	*M. lucifugus*	IC	14,000 mouse IC LD_{50}	20th	+	+	+	
4	*M. lucifugus*	IC	14,000 mouse IC LD_{50}	21st	+	+	−	
5	*M. lucifugus*	IM	Alaska wolf 35,000 mouse IC LD_{50}	20th	+	+	−	
6	*M. lucifugus*	IM	35,000 mouse IC LD_{50}	27th	−	+	+	
7	*M. lucifugus*	IM	35,000 mouse IC LD_{50}	33rd	−	+	−	28th, 32nd, & 33rd. All negative.
8	*M. lucifugus*	IC	Florida bat #250 0.29 mouse IC LD_{50}	25th	−	+	−	
11	*M. lucifugus*	IC	0.29 mouse IC LD_{50}	50th	+	+	+	32nd thru 50th. Positive 36th, 37th, 39th, 43rd, 48th, 49th.
17†	*M. lucifugus*	IM	Florida bat #61 M_1 17.0 mouse IC LD_{50}	Normal thru 126th				3rd thru 80th. All negative.
18†	*M. lucifugus*	IM	17.0 mouse IC LD_{50}	Normal thru 126th				3rd thru 80th. All negative.
17	*M. lucifugus*	IM	Alaska wolf 13,300 mouse IC LD_{50}	31st	+	+	−	3rd thru 30th. All negative.
18	*M. lucifugus*	IM	13,300 mouse IC LD_{50}	19th	−	+	−	3rd thru 18th. All negative.
19	*E. fuscus*	IM	Florida bat 682 M_1IL_1* 170 mouse IC LD_{50}	43rd	+	−	−	1st thru 43rd. All negative.

IC, intracerebral.
IM, intramuscular.
* 3 intracerebral and 1 intralingual passages in mice.
† Bats no. 17 and 18 remained normal for 126 days after inoculation with the Florida bat strain of virus. They were then inoculated with the Alaska wolf strain.

in the salivary glands at death, revealed a fairly consistent presence of virus during the 14-day period preceding death.

It was observed that for a given preparation of virus the period between inoculation and death was substantially longer in bats than in mice. The Alaska wolf material killed mice in 6 to 9 days, and bats in 10 to 33 days. Mice inoculated with the Florida bat strains died in 13 to 22 days, and bats died in 25 to 50 days. Five mouse passages of one Florida bat strain did not result in any change in the incubation period or in the production of Negri bodies.

Four bats in this series died after having been inoculated with rabies virus and developing symptoms suggestive of rabies. Brain smears of all but one were Negri negative and no virus was isolated from any of the brains or salivary glands. The process of autosterilization[13] may account for these findings.

13. Van Rooyen and Rhodes. 1948, Virus Diseases of Man, New York, Thomas Nelson and Sons, p. 287.

Bats were maintained without difficulty under the conditions of these experiments, being introduced to the laboratory both from the hibernating state and when fully active. Chopped meal worms, earthworms, and crickets were offered to the bats; all were eaten readily. Crickets were fed during most of the experiments because they could be obtained in large lots from a fish bait shop and stored in a refrigerator for several days without spoiling.

The species of bats used in these studies proved to be tractable. After a few days in captivity they become calm, somewhat obese, and did not attempt to escape. Large areas for exercise did not appear to be essential. Specimens of *M. lucifugus* were maintained in this manner for as long as 157 days, and *E. fuscus* for 65 days, but two specimens of *P. subflavus* died after 53 days from nonspecific causes.

DISCUSSION

Since the original isolation of rabies virus from insectivorous bats in Florida, isolations have also been made in Penn-

sylvania, Texas, California, and Montana. The only attempts at quantitation (the studies made in Florida[10] and Texas[12]) have revealed an impressively high incidence of infection. Thus it would appear that sufficiently efficient mechanisms of transmission exist for the maintenance of the disease among bats in endemic form over wide areas.

No records exist which suggest an active role for insectivorous bats in the epidemic form of rabies. Consideration of the natural habitat and habits of these bats would seem to minimize their potential for such a role. However, since bats constitute part of the normal diet of some carnivores, such as foxes, it would be well to investigate the possibility of predators becoming infected through the ingestion of rabid prey.

The unusually long incubation period in mice of rabies virus isolated from bats and the even longer incubation period of these strains in bats, suggest some modification of the virus. One would expect such a change to occur only after a host-virus relationship of long standing. Whether or not insectivorous bats may recover from rabies infection (a condition observed occasionally in vampire bats) was not determined with the limited number of bats used in this study.

SUMMARY

Insectivorous bats were successfully maintained under laboratory conditions.

These bats proved to be susceptible to preparations of rabies virus isolated from naturally infected bats and a wolf. Infection was produced by both intramuscular and intracerebral inoculation.

Symptoms displayed consisted of loss of appetite, weakness, tremors, irritability, muscular spasms, paralysis, biting at the site of inoculation, and aggressiveness.

The preparations of virus isolated from naturally infected bats had an unusually long incubation period in mice and an even longer incubation period in bats.

Rabies virus was repeatedly recovered from the saliva of one bat during the two-week period preceding death of the animal.

The implications of these findings are discussed.

THE PATHOGENIC COMPONENTS OF AN EXPERIMENTAL FUSOSPIROCHETAL INFECTION

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It was recently shown that infections having all the attributes of experimental fusospirochetal infections[1] could be produced regularly in guinea pigs with a combination of 17 different organisms—all isolated in pure culture from a guinea pig mixed infection.[2] Of the 17 organisms 15 were strict anaerobes and included streptococci, *Bacteroides*, spirochetes, vibrios and other motile rods, and nonmotile gram-positive rods, presumably diphtheroids; two were facultative and included a strain of *Streptococcus mitis* and a diphtheroid.

A new technique of recombination the "wheel-plate" was employed in the recombination. This technique consisted of inoculating a blood agar medium with each of the pure cultures as a single streak, starting at the edge of the plate and finishing in the center in such a way that on completion the streaks appeared as spokes of a wheel. After incubation each streak could be examined for evidence of pure growth of the inoculated strain and the hub of the wheel in successful plates contained mixed growth of all the organisms.

In the present study the mixed infection has been further investigated by deleting organisms from the recombinations to determine the minimum combination capable of eliciting typical guinea pig responses.

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1. Rosebury, T. and Foley, G. 1939, J. Am. Dent. A. **26**: 1798–1811.
2. Macdonald, J. B., Sutton, R. M. and Knoll M. L. 1954, J. Infect. Dis. **95**: 275–284.

1. ELIMINATION OF NONESSENTIAL ORGANISMS

Method

Wheel-plates were prepared in which various organisms were omitted, and guinea pigs were inoculated with mixed growth obtained by subculture from the wheel-plates following the same procedure as in the recombination of 17 organisms previously described.[2] Each recombination emulsion was inoculated into separate groups of four or more guinea pigs, 1 ml per dose. In those instances in which infection resulted, exudate was aspirated (usually from two of the four animals) and passed to additional animals in a dosage of 0.1 ml. In this way the infection was carried through four or more passages wherever possible. The lesions in all guinea pigs were scored from 0 to ++++, according to the procedure recorded in table 1.

All guinea pigs were maintained on a diet of Purina checker chow supplemented 3 times a week with fresh cabbage.

In initial experiments recombinations were prepared in which all strains within a morphologic group were omitted.* Subsequently individual strains within groups which appeared essential were omitted. The system of elimination of organisms was designed to give at least one confirming experiment where a group or an individual organism appeared in a first experiment to be indispensable.

The results were evaluated by analyzing for two attributes, infectivity and transmissibility. Infectivity was considered to depend on the ability of the mixed culture to produce infection in the first passage. The occurrence of infection in passages beyond the first was considered to be a measure of transmissibility. For each attribute the proportion of typical infections to the total

* Strain K100, a streptococcus, was omitted from all recombinations because earlier studies showed it to be unnecessary.[2]

number of animals inoculated in the experimental group was compared to the corresponding proportion in a control group. The control for this purpose was a group of 60 animals inoculated with the total recombination mixture in three separate experiments.[3]†

Results

The results are tabulated by groups arranged alphabetically as shown in table 2.* The findings in groups A to C inclusive suggested that spirochetes, cocci (six strains) and fusiforms could be omitted without interfering with the infectivity or transmissibility of the infection as judged by the proportion of typical infections in each group. The findings for these three groups were confirmed in subsequent groups, i.e., groups J, K, N, Q, and W. In only one group was there apparent conflict with this result, i.e., group G, in which there appeared to be a significant loss in transmissibility. In view of the repeated confirmations this discrepancy was regarded as not significant and possibly related to fluctuation of the numbers of organisms in the constant volume inoculums.

The results in groups D to F suggested a need for representatives of the gram-positive rods, the motile rods, and the *Bacteroides*. In groups H to J the three strains of *Bacteroides* were omitted singly, along with spirochetes and cocci. Losses in infectivity and transmissibility occurred in the absence of either of 2 strains (K110, K92) but not in the absence of the 3rd (JR6). The dispensability of the latter was confirmed in groups K, N, Q and W.

Among the 3 strains of gram-positive rods, strain JB3B appeared to be dispensable (group N). Omission of JB3A (group M) seemed to result in a loss of transmissibility, but this finding was not confirmed in groups Q and W. Omission of JR3 (the facultative diphtheroid) affected transmissibility (group L). When JR3 was omitted from wheelplates with smaller combinations than in group L, strain K110 (*Bacteroides nigrescens*) consistently failed to grow. By cross-streaking these 2 strains it was found that K110 grew as a satellite of JR3. The need for an unidentified "growth factor" for strain K110 was reported previously.[3] Cross-streaking strain K110 against all of the strains disclosed that in the presence of strains JR3, JR4, JR5 or JS9, strain K110 grew well. It was found to grow poorly in the presence of strain JB3B and not at all with any of the other strains. It therefore appears that one function of strain JR3 in the mixed infection was to support growth of strain K110.

Omission of the two strains of motile rods singly in groups O and P suggested that both were needed for typical infections. However, in subsequent combinations of the remaining five strains, four at a time, it was shown that one of them, JV5, could be omitted without evidence of change in infectivity or transmissibility.

Trial of all possible combinations of three of the four apparently essential

† The method did not take into consideration the severity of infection as indicated by the scoring + to ++++, but analysis of the results of inoculating 134 guinea pigs with unpurified exudate indicated that there was no significant relationship between the occurrence of failures (0, ±) and the severity of the the infection from which the inoculum was derived in the range + to ++++. Thirty-eight guinea pigs failed to develop infection when inoculated with exudate from localized lesions as against 61 which developed typical infection. Using exudate from spreading lesions, 8 guinea pigs failed to develop infection and 27 developed typical infection. Chi-square for 1 degree of freedom = 2.1.

* The probabilities of differences between experimental and control proportions are based on the exact binomial or in larger samples are derived from chi-square adjusted for continuity and symmetry.

organisms confirmed the above results. No infection developed in any of two groups of five guinea pigs each, inoculated with a combination in which one or other of the *Bacteroides* strains were omitted. Omission of the motile rods (K80) resulted in typical infection in the first passage (all of 5 animals) and no infection in the second passage (9 guinea pigs).

The results of these elimination studies suggested that a combination of not less than four organisms was essential to the production in the guinea pig of mixed infection comparable to the "fusospirochetal" infection produced with 17 organisms. The four strains apparently essential included two strains of *Bacteroides* (K110 and K92), one motile gram-negative rod (K80) and a facultative diphtheroid (JR3).

2. COMPARATIVE PATHOGENICITY OF THREE RECOMBINATION MIXTURES

On the basis of the above findings it was postulated that a combination of the four apparently essential organisms should produce infection in guinea pigs indistinguishable from that produced by the total group of 16* strains; and that the total group without the 4 essential organisms should be nonpathogenic. This hypothesis was tested. For convenience the three recombinations above are referred to as "four" (the essential 4, see table 2), "sixteen" (for total) and "twelve" (total less the essential 4 strains).

Method

Wheel-plates were prepared for the "four" group and the "twelve" group. The wheel-plate growth was emulsified in glass tissue grinders and subcultured as unstreaked drops to fresh blood agar in each instance. The growth from 12 drops of each was emulsified in 6 ml of broth. Three ml of the resultant emulsion of "four" were mixed with 3 ml of the emulsion of "twelve." This procedure mechanically reconstituted the total group of 16 organisms and was labelled "sixteen." Three ml of the remaining "four" emulsion were emulsified with 3 ml of an emulsion of sterile blood agar and broth prepared in the same way as the "four" and "twelve" emulsions. A similar mixture was prepared from the "twelve" emulsion and a sterile emulsion. The net result of this method of preparing the recombination mixtures was that the "four" and "sixteen" recombinations contained equivalent amounts of the recombination of the 4 apparently significant organisms; and the "twelve" and "sixteen" recombinations contained equivalent amounts of "twelve"—the fraction presumed to be nonpathogenic. In addition, the 3 recombination mixtures contained equivalent amounts of blood agar and broth.

Three groups of 5 guinea pigs matched for weight (300 to 360 g) were inoculated with the recombination mixtures, each animal receiving 1 ml of the appropriate emulsion subcutaneously in the groin. The animals were randomized in the cages and their positions changed daily. Their condition was recorded daily by one of us (JBM) who did not know to which group any of the animals belonged. Where possible, exudate was aspirated from appropriate lesions on or before the 7th day and passed to fresh animals in a dosage of 0·1 ml. This procedure was repeated through additional passages as shown in table 1.

Results

Typical and indistinguishable abscesses developed in all 5 animals inoculated with "sixteen" (total recombination) or "four" (minimum pathogenic) recombination (table 1). The abscesses were well walled off and contained abundant thin, creamy, foul smelling exudate, yellow or grey-brown in color. Sections from the walls of abscesses stained with hematoxylin and eosin disclosed a picture of acute necrotizing inflammation with a well-defined border between granulation and necrotic tissue. Animals inoculated with "twelve" (presumed nonpathogenic) fraction in each case developed only small fibrotic

* Strain K100, a streptococcus, was omitted from all recombinations, because earlier studies showed it to be unnecessary.

TABLE 1.—*Pathogenicity of different recombination mixtures in guinea pigs.*

Passage	Inoculum	"16" Group	"4" Group	"12" Group
1	1 ml cult. mash	+*, +*, +, +, ++	+*, +*, +, +, +	±, ±, ±, ±, ±
2	0.1 ml exudate	+, +, ++*, ++	+*, +, +, +	
3	0.1 ml exudate	+, +, ++, ++	±, ±, ±, +*	
4	0.3 ml exudate		++*, +++	
5	0.3 ml exudate		++*, +++	
6	0.3 ml exudate		++*, ++	
7	0.1 ml exudate		+++, ++++	

±, small nodular lesion; little or no exudate.
+, less than 1 inch localized abscess.
++, localized abscess 1 inch or more in diameter.
+++, localized abscess with extension; not fatal in 7 days.
++++, spreading infection fatal in 7 days or less.
Each symbol represents 1 guinea pig.
* Exudate used for passage.
"16," total recombination.
"4," presumed pathogenic fraction.
"12," presumed nonpathogenic fraction.

nodules with no exudate. Passage to additional animals therefore was not possible within this group.

Typical abscesses developed in all 8 animals of the 2nd and 3rd passages in the "sixteen" group and in 5 of 8 guinea pigs in the "four" group. There was an apparent decrease in infectivity of the exudate used for 3rd passage inoculation in "four" group (only 1 typical abscess in 4 animals), but typical abscesses and also spreading lesions occurred in 4 subsequent passages of the "four" group (table 1).

All exudates were examined by darkfield. In the case of exudates derived from "sixteen" the appearance was typical of fusospirochetal material; those from "four" disclosed only rods, most of which were nonmotile. Exudates from 10 animals in various passages of the "four" recombination were streaked on blood agar and incubated aerobically and anaerobically for 7 days. In every instance the original 4 organisms (strains K110, K92, JR3 and K80) were recovered without evidence of contamination.

DISCUSSION

The distinction between infectivity and transmissibility on the basis of capacity to produce infection in the first as opposed to subsequent passages is in a sense artificial. The first passage was suitable for measuring infectivity. Under the conditions of these experiments, transmissibility was a measure of the capacity of the infective agents to persist in the exudate, i.e., a measure of the retention of infectivity. Thus the results in table 2 suggest that in certain instances infectivity differed between first and subsequent passages. Discounting the instances in which individual experiments appeared to be in conflict with the over-all results (groups G and O), it can be seen that omission of either JR3 or K80 resulted in a loss of infectivity (transmissibility) beyond the first passage, whereas in the first passage typical infection did occur in the absence of either of these organisms. The role of strain JR3 as noted is apparently directly related to the maintenance of strain K110. The role of strain K80 in maintaining infectivity is not known.

The final experiment offers convincing evidence that only a small fraction of the complex mixed flora is required to produce typical infections and that the majority of the organisms play no essential role. The findings in the first passage in this experiment (table 1) are the only ones which are strictly comparable. The inoculum in each of the 3 groups (pathogens versus total, and total versus nonpathogens) were made equivalent in respect to bacterial con-

tent and the findings are clear-cut. Infection developed in every animal where the pathogenic quartette was present in the inoculum and in none of 5 animals where only the remaining 12 organisms were injected. Direct comparisons beyond the first passage are hazardous because no data are yet available concerning fluctuations in bacterial count from exudate to exudate. There is therefore no assurance that dosage in terms of numbers of viable organisms was comparable beyond the first passage. Nevertheless, the results demonstrate further that transmissible infection indistinguishable from unpurified fusospirochetal infection can be produced with a combination of only 4 of the 17 organisms composing the total flora of this particular exudate.

The minimum combination of organisms capable of eliciting typical "fusospirochetal" responses in the guinea pig as shown in these studies is not necessarily the only combination which would be effective. For example, it is possible that one or other of the 4 organisms found to be significant could be replaced by one or other of the several strains of cocci or by the spirochetes. That interchange of certain of these organisms might be possible is suggested by the observation that several of the organisms produce the "growth factor" required for strain K110.

The striking finding in these experiments is that infection can be produced with a combination of organisms devoid of both spirochetes and fusiforms. Likewise, cocci and vibrios were nonessential in this instance, although representatives of all of the above 4 types were reported necessary by both Smith[3] and Proske and Sayers.[4] On the other

TABLE 2.—*Recombination mixtures used for guinea pig inoculation.*

Group	Composition of recombination mixtures										Proportion typical infection		Probability values infectivity transmissibility	
	Spirochetes	Cocci	Fusiform JF3	Gram-positive rods JR3	JR3A	JR3B	Motile rods JV5	K80	K110	Bacteroides K92	JR6	1st Passage	2–5 Passage	1st Passage	2–5 Passage
Control	+											12/12	46/48	0.50	0.78
A		++	+++	++++	++++	++++	++++	+++++	+++++	+++++	+++	3/4	7/9	1.00	>0.99
B	+	++++	+++++++	+++++++	+++++++	+++++++	+++++	+++++	+++++	+++++	+++	4/4	11/12	0.50	0.55
C												3/4	14/16	0.50	<0.01
D												3/4	8/15	0.01	<0.01
E												1/4	1/8	1.00	<0.01
F												6/6	0/3	<0.01	<0.01
G												0/4	0/3	<0.01	<0.01
H												4/4	9/16	1.00	<0.01
I												0/4		<0.01	
J												0/4	0/2	0.10	<0.01
K												2/4	14/16	0.50	0.55
L												3/4	13/16	0.50	0.14
M												3/4	5/12	0.50	<0.01
N												3/4	8/12	0.50	<0.05
O												4/4	16/17	1.00	<0.01
P												4/4	0/3	1.00	<0.01
Q												4/4	0/4	1.00	<0.01
R												4/4	16/16	1.00	>0.99
S												4/4	2/15	1.00	<0.01
T												1/5		<0.01	
U												0/4		<0.01	
V												2/5	0/5	0.01	<0.01
W												4/5	25/28	0.65	0.53

3. Smith, D. T. 1930, J. Infect. Dis. **46**: 303–311.
4. Proske, H. O. and Sayers, R. R. 1934, Pub. Health Rep. **49**: 839–858; 1212–1217.

hand, Kritchewski and Seguin[5] reported that typical infections in the guinea pig resulted from combinations of spirochetes, fusiforms and either a greening streptococcus or a white staphylococcus. The divergency of these results supports the view that these mixed infections may be nonspecific in a bacteriologic sense. The uniformity of the lesions produced from a variety of sources does suggest, however, a biochemical specificity—a need for a particular combination of metabolites, toxins or enzymes not produced by any one organism.

5. Kritchewski, B. and Seguin, P. 1920, Rev. di Stomatol. **22**: 41; 613–647.

SUMMARY

The pathogenicity of an experimental fusospirochetal exudate has been investigated in terms of the minimum combination of organisms capable of producing typical guinea pig infections. From a total of 17 organisms isolated in pure culture, only 4 were required to produce typical lesions. The 4 essential organisms included 2 strains of *Bacteroides* (one of which was identified as *Bacteroides nigrescens*), a motile gram-negative anaerobic rod and a diphtheroid. Spirochetes, fusiforms, vibrios and anaerobic streptococci were found to be nonessential.

SUSCEPTIBILITY TO INFECTION AS INFLUENCED BY ACCLIMATIZATION TO ALTITUDE AND KREBS CYCLE INHIBITORS AND INTERMEDIATES

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Exposure of mice to simulated high altitude of about 20,000 feet for a period of 3 weeks renders them more susceptible than normal control mice to artificially induced infections with *Salmonella typhimurium*.[1] No experimentally detectable differences in either the cellular or the humoral defense mechanisms, however, could be found to accompany adaptation to altitude. It was postulated that the greater susceptibility of such mice was due to an undetected and unspecified type of alteration in metabolism.

In order to test the validity of the suggestion that host metabolism modifies response to infection, the survival time of normal mice infected with *S. typhimurium* was compared to that of mice similarly infected and then given a series of injections of compounds known to inhibit certain steps in the tricarboxylic acid cycle.[2,3] Intermediates of the cycle also were employed. Under the conditions of the experiments, several of these compounds decreased survival time significantly below that of control animals. In infections with bacterial pathogens other than the organism of mouse typhoid, injections of at least one of these substances, but not always the same, killed the animals more quickly than infection alone.[4]

The present report is concerned with the comparative susceptibility of altitude mice and of normal mice, infected with either *S. typhimurium* or *Diplococcus pneumoniae*, and then injected with inhibitors or intermediates of the Krebs cycle.

METHODS

Mice were subjected to simulated altitude of about 20,000 feet according to the method previously described.[1] They were exposed for a period of 3 weeks immediately prior to infection.

Mice were infected intraperitoneally with 0.5 ml of a saline suspension of *S. typhimurium* containing about 250,000 cells. This was prepared immediately before use by appropriate dilution of a 15-hour brain heart infusion broth (Difco) culture. In those experiments in which mice were infected with pneumococci, the suspension was prepared by diluting a 15-hour blood broth culture of *D. pneumococci* (SV-1) 1:10,000,000 with nutrient broth. Each animal was injected intraperitoneally with 0.5 ml of the final dilution, approximately 50 cells.

Compounds used to artificially "modify" the metabolism of mice were: disodium malonate (Eastman), sodium monofluoroacetate (kindly supplied by Monsanto Chemical Company), sodium arsenite (Merck reagent), trisodium citrate (Merck reagent), disodium succinate (Merck), and sodium oxaloacetate (Nutritional Biochemical Corporation). Each substance was

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1. Berry, L. J. and Mitchell, R. B. 1953, Texas Rep. Biol. & Med. **11**: 379–401.
2. Berry, L. J. and Mitchell, R. B. 1953, J. Infect. Dis. **93**: 75–82.
3. Berry, L. J. and Mitchell, R. B. 1953, J. Infect. Dis. **93**: 83–92.
4. Berry, L. J., Merritt, P. and Mitchell, R. B. 1954, J. Infect. Dis. **94**: 144–151.

dissolved in sterile saline so as to give the following sublethal amounts in 0.5 ml of the final solutions: malonate, 20 mg; fluoroacetate, 0.16 mg; arsenite, 0.125 mg; citrate, 10 mg; succinate, 20 mg; and oxaloacetate, 10 mg. Since all animals weighed 20 g ±2 g, the dosage per unit weight is readily estimated.

Two groups of control mice and 2 groups of altitude mice, each consisting of 5 animals, were established in separate cages for each of the compounds to be administered. Altitude mice were removed from the decompression chambers about 1 hour prior to infection. A group of ground level mice and a group of altitude mice were infected intraperitoneally and immediately thereafter injections of metabolic inhibitor or intermediate were begun. Similar injections also were administered to the 2 uninfected groups which served as toxicity controls. Animals given malonate, citrate, and succinate were injected at hourly intervals for a total of 8 injections. Those receiving oxaloacetate were injected every 2 hours for a total of 4 injections. The second injection of arsenite was given 1 hour after the first, and the third, 1½ hours after the second. Two injections of fluoroacetate were administered, the second following the first by 6 hours. One group of ground level mice and one group of altitude mice were infected along with the other groups and were given hourly injections of 0.5 ml of sterile saline for a total of 8 injections. These served as controls for the infection. There is abundant evidence to prove that the course of the infection is not significantly altered by repeated injections of saline.

Three separate experiments were carried out with each of the two pathogens. Female CF 1 mice were used throughout.

RESULTS

1. *Comparative effect of inhibitors and intermediates of Krebs cycle on survival time of altitude mice and ground level mice infected with* S. typhimurium.—Table 1 summarizes the results of 3 separate experiments. Attention is directed to the fact that altitude mice are more susceptible than ground level mice to *S. typhimurium*, as shown by the row designated "saline." This confirms previous results.[1] At 144 hours postinfection, beyond which no deaths occurred, only 1 of 15 altitude mice and 9 of 15 control mice survived. This is significant at less than the 0.01 level, as determined by the rank order test of White.[5] In calculating the mean survival times of the animals in all groups, those surviving the infection were considered to have survived for only 144 hours. This becomes meaningless in absolute terms only in the case of the ground level "saline" controls but for statistical purposes it represents the least favorable condition for calculating significant differences. In all other groups, no more than one animal survived and the error represented is, therefore, comparatively small. It should be noted, however, that the survival time of the ground level animals is longer than has been the case in earlier work,[6] suggesting that the culture of *S. typhimurium* has undergone a decrease in virulence. This is further borne out by the longer survival times seen in mice injected with certain ones of the 6 compounds listed, compared with reported data.[6]

TABLE 1.—*Mean survival time in hours of mice* following intraperitoneal infection with 250,000 cells of* S. typhimurium *followed by a series of injections of inhibitors or intermediates of the citric cycle. (Estimated standard deviation = 41.2).*

Inhibitor or intermediate	At altitude† (A)	At ground level (N)	Total means	Altitude effect (A − N)
Malonate	54.9	75.1	65.0	−20.2
Succinate	51.9	76.2	54.0	−24.3
Citrate	46.1	91.3	68.7	−45.2
Fluoroacetate	42.5	43.3	42.9	− 0.8
Oxaloacetate	51.1	84.4	67.8	−33.3
Arsenite‡	26.0	109.2		
Saline	95.7	128.4	112.0	−32.7
Total Means	57.0	74.2	65.6	−17.2

* 5 mice used in each of the 2 arsenite groups. 15 mice used in each of the other 12 groups. These 15 mice are a combination of 3 separate experiments each with 5 mice.

† Mice kept at stimulated 20,000 feet for 3 weeks prior to infection.

‡ Values for arsenite not included in total means nor in analysis of variance (table 2).

Further study of table 1 reveals that for mice exposed to altitude the mean survival time is significantly less than that for mice kept at ground level when

5. White, C. 1952, Biometrics, **8**: 33–41.
6. Berry, L. J. and Mitchell, R. B. 1954, J. Infect. Dis. **95**: 246–259.

all groups are combined (57 hours versus 74.2 hours). This is consistent for all compounds employed ($P<0.001$ by analysis of variance). It is seen also that considerable variation in survival time occurs among the different altitude groups. These differences are statistically significant as shown by analysis of variance ($P<0.001$). The last column of table 1 denotes that the effect of altitude is not apparently the same with each of the groups injected with one of the compounds. This variation is statistically significant at about the 0.05 level and may be tentatively accepted as a valid characteristic of adaptation to altitude.

Besides knowing that the groups given the different substances vary in general, it is desirable to assess this variation more carefully. Excluding arsenite (for reasons revealed by the data in table 3) and using a multiple range test,[7] it is found that, with a significance of 0.05, (1) both at ground level and at altitude the saline group survives longer than all other groups, and (2) at ground level but not at altitude the fluoroacetate group survives for a shorter time than the group given oxaloacetate. The other groups do not differ significantly.

In evaluating the effect of altitude, it is found that injections of oxaloacetate, citrate, saline, and arsenite each yield, according to the t test, significantly shorter survival times in altitude mice than in ground level mice. The remaining compounds do not give significant differences in this respect.

2. *Comparative effect of inhibitors and intermediates of the Krebs cycle on survival time of altitude mice and ground level mice infected with pneumococci.*— Table 2 presents the combined results of 3 separate experiments. As shown by analysis of variance, the differences obtained among the several treatments listed in the first column of table 2 are significant ($P<0.001$). The one outstanding observation is that of malonate at altitude. Here the mean survival time is significantly lower than for any of the other 13 groups. By use of the multiple range test,[7] it can be stated that at altitude, treatment with malonate, arsenite (but see table 3), fluoroacetate, or succinate leads to significantly lower survival times than treatment with saline or oxaloacetate. Similarly, the same situation prevails when malonate and arsenite injected animals are compared with those given citrate. At ground level, all compounds, except citrate, when injected lead to shorter survival times than saline-injected mice, while those given fluoroacetate die significantly sooner than the group given citrate. At both altitude and ground level, the group injected with saline has a significantly higher survival time than those given malonate, fluoroacetate, succinate, and arsenite.

At altitude, the survival time is lower than at ground level but with borderline significance (the P value is

TABLE 2.—*Mean survival time in hours of mice* following intraperitoneal infection with about 50 cells of* D. pneumoniae *(SV-I) followed by a series of injections of inhibitors or intermediates of the citric acid cycle. (Estimated standard deviation = 17.8).*

Inhibitor	At altitude† (A)	At ground level (N)	Total means	Altitude effect (A − N)
Malonate	20.8	42.6	31.7	−21.8
Succinate	36.6	40.8	38.7	− 4.2
Citrate	49.5	50.9	50.2	− 1.4
Fluoroacetate	38.4	34.9	36.6	+ 3.5
Oxaloacetate	52.5	44.3	48.4	+ 8.2
Arsenite	34.5	40.7	37.6	− 6.2
Saline	53.8	64.9	59.1	−11.1
Total Means	41.5	46.1	43.8	− 4.6

* 5 mice used in each of the two arsenite groups. 15 mice used in each of the other 12 groups. These 15 mice are a combination of three separate experiments each with 5 mice.

† Mice kept at simulated 20,000 feet for 3 weeks prior to infection.

7. Duncan, D. B. 1952, Tech. Rep. no. 6, Dept. of Statistics, Va. Polytechnic Inst., Blacksburg, Va.

0.05 or more depending upon the test employed). This is not consistent, in fact it is significantly not consistent, from substance to substance. Most striking is the rise in survival time of the altitude group given oxaloacetate and to a lesser extent fluoroacetate as contrasted with the strong fall in survival time of the group given malonate. Significant altitude effects occur only with the malonate group.

3. *Comparative effect of inhibitors and intermediates of the Krebs cycle on survival time of uninfected altitude mice and ground level mice.*—Table 3 presents the combined results of 4 separate experiments in which uninfected altitude mice and uninfected control mice were given the same series of injections of inhibitors and intermediates as infected mice described in the sections above. It is apparent that arsenite is much more toxic for altitude mice than it is for ground level mice. By the rank order test[5] the difference is statistically significant at the 0.01 level. The same statistical significance is found for the greater susceptibility of altitude mice injected with malonate even though these animals show a smaller percentage mortality. Death of the one normal mouse given malonate was perhaps due to a peritoneal hemorrhage observed at autopsy. This was only the third animal, of the several hundred injected with this substance over a period of months, to succumb, and in all cases death was apparently attributable to trauma rather than to malonate toxicity.

Fluoroacetate killed none of the altitude mice and only three of the control animals. This difference is not statistically significant. Injections of the three intermediates, as mentioned in the footnote to table 3, were not accompanied by any deaths of the mice in these groups.

TABLE 3.—*Number of mice surviving at times indicated during and after the administration of a series of intraperitoneal injections of inhibitors* of the citric acid cycle.*

Time after first injection (in hours)	Number of mice surviving injections of					
	Malonate 8 ×20 mg		Fluoroacetate 2 ×0.16 mg		Arsenite 3 ×0.125 mg	
	Altitude	Normal	Altitude	Normal	Altitude	Normal
0	38	31	30	30	20	20
5	35	31	30	30	15	19
10	25	30	30	30	10	19
24	25	30	30	27	7	19
38	25	30	30	27	6	19
144	25	30	30	27	6	19

Response of mice that had been kept at simulated 20,000 feet for 3 weeks and at atmospheric pressures for about 1 hour prior to the first injection is compared with that of normal control mice. *None of the mice were infected.* The table presents the combined results of 4 separate experiments.

* Altitude and normal mice were injected also with succinate (8 ×20 mg), citrate (8 ×10 mg), and oxaloacetate (4 ×10 mg) but none of the animals died. There were 20 mice in each of the individual groups, the combined results of 4 separate experiments.

DISCUSSION

In order to study more closely the various interrelationships among injected compounds, altitude, and infection, the percentage of animals surviving at 36 to 38 hours postinfection was tabulated. Data leading to tables 1, 2, and 3 were used to construct table 4. The effect of inhibitors and intermediates was examined by subtracting the mean percent of mice surviving each compound, as found in the first 6 rows of table 4, from the percent surviving saline shown in the last row of the same table. Doing this for each column con-

TABLE 4.—*Percent of mice surviving at 36 to 38 hours by inhibitor, type of infection, and altitude.*

Inhibitor	Percent of animals in each group surviving					
	No infection		*S. typhimurium*		*D. pneumoniae*	
	At altitude	At ground level	At altitude	At ground level	At altitude	At ground level
Malonate	66	97	47	73	0	40
Succinate	100	100	47	73	20	40
Citrate	100	100	47	80	53	60
Fluoroacetate	100	90	53	67	27	40
Oxaloacetate	100	100	53	93	80	67
Arsenite	30	95	20	100	20	80
Saline	100	100	93	100	93	100

TABLE 5.—*"Inhibitor effect" at altitude and at ground level with no infection and with infection with* S. typhimurium *and with* D. pneumoniae *(SV-1).*

Comparison	No infection		*S. typhimurium*		*D. pneumoniae*	
	At altitude	At ground level	At altitude	At ground level	At altitude	At ground level
Percent surviving saline minus mean percent surviving inhibitors	17%	3%	48%	19%	60%	42%
Percent surviving saline minus percent surviving arsenite	70%	5%	73%	0%	73%	20%
Percent surviving saline minus percent surviving malonate	34%	3%	46%	27%	93%	60%
Percent surviving saline minus mean percent surviving inhibitors excluding arsenite	7%	3%	44%	23%	57%	51%

taining "percent surviving," the values representing "inhibitor effect" were obtained as presented in table 5.

It is important to note that this "inhibitor effect," while somewhat enhanced at altitude when there is no infection, is much larger at altitude than at ground level in the presence of *S. typhimurium* and *D. pneumoniae*, except in the presence of arsenite alone. Thus the effect of this substance appears to be dependent upon the results of the hypoxic stress alone and not upon the combination of altitude and infection (row 2 of table 5). If the effects of altidue and inhibitor were simply additive, the altitude minus ground level values obtained from uninfected mice should be the same as those obtained from infected ones, as it was with arsenite. When the result is contrary to this expectation, it is indicative of the existence of some kind of interaction which leads to greater sensitivity to infection than one would expect in the combined presence of inhibitors at altitude. This is particularly important in the studies with malonate, which alone kills about one-third of the altitude mice but significantly more than this when they are infected, particularly with pneumococci. A test of the statistical significance of the above is difficult to apply. A rough test was possible and indicated the results were of borderline significance (P about 0.05) with respect to the first and third rows of table 5. The other rows did not provide significant findings.

These observations support the concept that mice undergoing acclimatization to altitude experience a change in metabolism, at least to the extent of greater sensitivity to certain poisons. Such mice also respond differently from ground level animals to artificially induced infections with and without subsequent injections of metabolic inhibitors and intermediates. The extent to which the two types of observations is causally related remains for future investigations to determine.

SUMMARY

1. Groups of CF 1 female mice that had been exposed to simulated altitude of 20,000 feet for 3 weeks and groups of ground level mice were infected intraperitoneally with *Salmonella typhimurium*. Immediately thereafter, a series of injections either of one of several inhibitors or intermediates of the Krebs cycle or of saline alone was administered each group. Both groups given saline survived significantly longer than the corresponding groups given any one of the compounds. Altitude mice survived significantly shorter times than ground level mice when they were injected with oxaloacetate, citrate, arsenite (see below), or saline.

2. In similar groups of mice infected with pneumococci, altitude mice in-

jected with malonate had a mean survival time significantly lower than that of any other group. No other differences of significance were found between altitude and ground level mice. Comparisons between mice given saline and those given one of the inhibitors or intermediates, revealed that for altitude mice, shorter survival times accompanied injections of fluoroacetate, succinate, or arsenite (see below), while at ground level only citrate failed to reduce survival time.

3. Groups of uninfected altitude and ground level mice were injected with the same substances as the infected mice described above. Arsenite killed 14 of 20 altitude mice and 1 of 20 normal mice ($P = 0.01$), indicating that the results with this substance in infected mice is due to hypoxic stress alone. This is not true for malonate, even though it killed 13 of 38 altitude mice and 1 of 31 normal mice ($P = 0.01$). Injections of fluoroacetate killed 3 of 30 normal mice and none of 30 altitude mice (difference not significant). All mice survived injections of citrate, succinate, and oxaloacetate.

THE PREPARATION OF POLIOMYELITIS COMPLEMENT-FIXING ANTIGENS FROM INFECTED TISSUE CULTURE FLUIDS

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The development of methods for preparing satisfactory and easily-made antigens has been a major problem in the application of complement fixation as a diagnostic tool in poliomyelitis. Preceding reports, particularly one by Svedmyr, Enders, and Holloway,[1] showed that fluids from tissue cultures infected with poliomyelitis viruses contained antigens which were satisfactory, although the technical procedures were rather involved. Recently the use of monkey kidney epithelial and HeLa cell cultures has made it possible to obtain fluids containing high titers of poliomyelitis virus when cells were infected with each of the three recognized immunologic types. This paper reports on studies made in this laboratory to prepare antigens from fluids derived from tissue cultures infected with several strains of poliomyelitis viruses.

MATERIALS AND METHODS

Viruses.—The following strains of viruses were used: type 1, Brunhilde and Pizarro;* type 2, Yale SK, Rodgers,* and Brooks; type 3, Leon, Saukett, Mabie, Eberly, and Dolislager. Each of these strains, excepting Rodgers and Pizarro, has been studied and reported on previously.[2]

Antiserum.—The standard type specific poliomyelitis antiserums prepared in this laboratory under auspices of the Typing Committee of the National Foundation for Infantile Paralysis were used. The antiserums contained complement-fixing activity as follows: type 1, serum dilution 1:64; type 2, serum dilution 1:256; and type 3, serum dilution 1:64.[3]

Tissue culture methods.—Three tissue culture systems were used in the preparation of antigens. The earliest studies were made with infected fluids derived from monkey testicular implants grown in Roux flasks.[4] Later HeLa cell cultures[5] and dispersed (trypsinized) monkey kidney cell cultures[6] were used. Methods of inoculation of tissue cultures, harvest time, and treatment of

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* We are indebted to J. F. Enders for the Pizarro and Rodgers strains of poliomyelitis viruses.

1. Svedmyr, A., Enders, J. F. and Holloway, A. 1952, Complement fixation with Brunhilde and Lansing poliomyelitis viruses propagated in tissue culture. Proc. Soc. Exper. Biol. & Med. **79**: 296–300.

2. Wenner, H. A., Miller, C. A. and Wilson, J. C. 1953, Immunologic classification of poliomyelitis viruses. VI. Results of typing by neutralization of prototype viruses with antiserum for the unknown strain. A. Studies from the University of Kansas on 67 strains. Am. J. Hyg. **58**: 52–80.

3. Wenner, H. A., Miller, C. A., Kamitsuka, P. and Wilson, J. C. 1954, Preparation and standardization of antiserums prepared with the three known types of poliomyelitis viruses. Am. J. Hyg. **59**: 221–235.

4. Miller, C. A. 1953, Growth of poliomyelitis viruses in large stationary cultures. Proc. Soc. Exper. Biol. & Med. **82**: 450–454.

5. Scherer, W. F., Syverton, J. T. and Gey, G. O. 1953, Studies on the propagation *in vitro* of poliomyelitis viruses. IV. Viral multiplication in a stable strain of human epithelial cells (strain HeLa) derived from an epidermoid carcinoma of the cervix. J. Exper. Med. **97**: 695–710.

6. Youngner, J. S. 1954, Monolayer tissue cultures. I. Preparation and standardization of suspensions of trypsin-dispersed monkey kidney cells. Proc. Soc. Exper. Biol. & Med. **85**: 202–205.

fluids containing poliomyelitis virus are described in detail later in this paper.

The complement fixation test.—Tests were performed using the plate method described by Fulton and Dumbell, with modifications.[7,8] All antigens were tested in "box titrations," using serial twofold dilutions of antigens and serial twofold dilutions of serum. Examples of box titrations using three antigens prepared from the Brunhilde strain of poliomyelitis virus are shown in table 1. Preliminary titrations of complement were done in the presence of antigens. In the test proper, two 100% units of complement were used. Antigens were heated at 56 C prior to use. Antiserums were heated for 20 minutes at 60 C. Antigens and antiserums, as well as other reagents, were diluted with veronal buffer.[9] The hemolytic system consisted of equal parts of a 0.4% sheep red blood cell suspension and a 1:2000 dilution of antisheep hemolysin containing between 6 and 8 hemolytic units. The hemolytic system was incubated for 1 hour at 37 C prior to use in the test.

Appropriate mixtures of serum, antigen, and complement were made on the plate and incubated in the refrigerator (4 C) overnight. A second complement titration was done in the test proper. Following incubation at 4 C, plates were removed and allowed to stand at room temperature for 10 minutes. Then the hemolytic system was added and the test system incubated at 37 C for 30 minutes. The plates were then removed from the incubator to room temperature. After standing 10 minutes, each plate was tapped gently to bring eccentric red blood cells into a button at the center of the drop. Readings were made on an illuminated frosted glass and recorded as 0, 1+, 2+, 3+, or 4+, 0 indicating complete hemolysis and 4+ indicating no hemolysis. The highest dilution of antigen or antiserum giving 2+ fixation was considered the serum or antigen end point titer. Antigen, serum, cell, and hemolytic controls were included in each test.

7. Fulton, F. and Dumbell, K. R. 1949, The serological comparison of strains of influenza virus. J. Gen. Microbiol. **3**: 97–111.
8. Morgan, P., Carter, J. S. and Wenner, H. A. 1953, An apparatus employed in plate complement fixation tests. J. Lab. & Clin. Med. **42**: 655–656.
9. Mayer, M. M., Osler, A. G., Bier, O. G. and Heidelberger, M. 1946, The activating effect of magnesium and other cations on the hemolytic function of complement. J. Exper. Med. **84**: 535–548.

RESULTS

Concentration of antigens by ultrafiltration.—The results of a box titration made with a concentrated type 1 poliomyelitis virus (Brunhilde) antigen appear in table 1, part A. Other tests followed this general pattern rather uniformly. A summary of results obtained in tests, many in replicate, with concentrated antigens representing the three immunologic types of poliomyelitis viruses appears in table 2.

Satisfactory complement-fixing antigens were prepared by the candle ultrafiltration method described by Svedmyr et al.[1] Roux flasks containing satisfactory outgrowths of fibroblasts derived from minced monkey testes were employed. Approximately 1000 $TCID_{50}$'s of virus were inoculated into a culture containing 75 ml of medium. Growth curves for Brunhilde, Yale SK, and Saukett viruses reached maximum infectivity titers of 10^{-3} to 10^{-4} (in testis tissue flask cultures) at 9-day harvests.[4] The following strains of poliomyelitis viruses were used: Brunhilde (type 1), Yale SK (type 2), and Leon and Saukett (each type 3). Fluids containing virus were harvested at 3-day intervals from about the 6th to about the 12th day after inoculation. The fluids were stored in a dry ice chest. Eventually fluids containing the same virus type were pooled and concentrated 100 to 600 times by ultrafiltration.

Satisfactory antigens were prepared with each of the four strains of viruses used. The antigen titers achieved were usually sufficient to permit a 1:4 dilution of antigen for use in the standard complement fixation test. The serum titers using diluted antigens prepared by ultrafiltration were deemed at the time workable although not necessarily optimal.

It was considered that weak responses were caused perhaps in pooling fluids

TABLE 1.—*Box titrations with Brunhilde antigens and type 1 antiserum.*

Antigen dilution	Complement fixation—serum dilution									Antigen control	Antigen titer	Serum titer
	4	8	16	32	64	28	256	512	1024			
A. Grown in monkey testicular tissue concentrated 200 times by ultrafiltration.												
2	4	4	4	4	4	2	1	±	0	0		128
4	4	4	4	4	3	1	0			0		
8	4	4	2	2	1	±	0			0		
16	3	1	±	0						0	16	
32	±	0								0		
64	0									0		
Serum control	0											
1.75 units complement in the test. Cell control 4+. Hemolytic control 0.												
B. Grown in HeLa cell tissue culture. Two-day harvest, unmodified.												
0	4	4	4	3	3	2	2	0		0		256
2	4	4	4	4	2	1	1	0		0	2	
4	1	0								0		
Serum control	0											
1.75 units complement in the test. Cell control 4+. Hemolytic control 0.												
C. Grown in dispersed monkey kidney cells. Two-day harvest, unmodified.												
0	4	4	4	4	4	4	0			0		128
2	4	4	4	4	4	3	1	0		0		
4	4	4	4	4	3	2	±	0		0		
8	2	2	±	±	0					0	8	
16	±	0								0		
Serum control	0											
2.0 units complement in the test. Cell control 4+. Hemolytic control 0.												

that contained varying amounts of antigen. Accordingly, a single experiment was designed in which fluids were harvested from separate bottles on days 3, 6, 9, and 12. Yale SK, a strain of type 2 poliomyelitis virus, was used. The concentrated fluids prepared from the fluid harvests were unreactive until sometime between the 6th and 9th days, when an antigen titer of 8 and a serum titer of 64 were obtained. Unforunately, the 12th-day fluid was anticomplementary.

Antigens derived from HeLa cell type cultures.—The observation that certain strains of poliomyelitis viruses reach TG_{50} values between 10^{-6} and 10^{-9} in trypsinized HeLa stationary tubes prompted us to try such infected fluid as antigen in the complement fixation test. Pilot studies were done with fluids derived from HeLa cell type cultures infected with several poliomyelitis virus strains. Variable results were obtained; some strains provided complement-fixing antigens, but some did not. Experiments were then designed to determine optimal times of harvest.

HeLa cells were grown in test tubes containing 0.4 ml or in bottles containing 8 ml of the nutrient medium defined

TABLE 2.—*Summary of results obtained with complement-fixing antigens prepared by ultrafiltration.*

Type	Strain of virus	Pool no.	Harvest times (days)	$TCID_{50}$	Times conc.	Antigen titer	Antigen titer* / serum titer	Heterotypic reactions
1	Brunhilde	1	7, 9, 12		600	32	4/64	0
		2	7, 9, 12		200	32	8/32	0
		3	7, 9, 12		200	16	2/128	0
2	Y-SK	1	13		100	4	4/8	0
		2	6		100	16	4/64	0
		3	3	$10^{-2.5}$	100	0	0/0	0
			6	10^{-4}	100	0	0/0	0
			9	10^{-4}	100	8	2/64	0
			12	10^{-5}	100	AC†		0
3	Leon	1	7, 10, 13		100		4/32	0
	Saukett	1	7, 10, 13		100		2/64	0

* Serologic reaction ratio: reciprocal of antigen titer/reciprocal of serum titer.
† Anticomplementary antigen.

by Scherer and Syverton.[8] The cultures were washed and maintenance medium was added. The tubes were then inoculated with 0.1 ml of 10^{-1} dilution of each of several virus strains. Bottles were also inoculated with 1.0 ml of the same dilution of virus. The following strains were used: type 1, Brunhilde and Pizarro; type 2, Yale SK, Rodgers, and Brooks; type 3, Leon, Mabie, Eberly, and Dolislager. These strains achieved titers ranging from 10^{-6} to 10^{-9} in HeLa cell cultures.

Twenty tubes containing satisfactory sheets of HeLa cells were inoculated with one of the strains of viruses listed above. Four tubes were harvested daily from the 2nd through the 6th day. The fluids were studied without any treatment other than light centrifugation. An example of a box titration with Brunhilde antigen appears in table 1, part B.

Some variation was found in regard to time of harvest of antigen for optimal activity. Cultures infected with type 1 viruses gave good antigens in fluids harvested as early as the 2nd day. Yale SK, a type 2 strain, reached an effective antigenicity on the 4th and 5th days. Leon, a type 3 strain, required a growth period of 3 to 4 days. In general, fluids harvested on the 4th and 5th days after inoculation gave the best results. Eberly and Dolislager, type 3 strains, titering 10^{-8} and 10^{-9} in HeLa cultures, failed to show complement-fixing antigenic activity. Some of the fluids harvested from the Brunhilde, Pizarro, and Rodgers strains of viruses were anticomplementary when used undiluted. The anticomplementary activity was lost in two- and fourfold dilutions of antigen.

Poliomyelitis complement-fixing antigens with antigenic titers of 1:2 to 1:8 and serum titers of 1:64 to 1:256 were obtained in HeLa tissue culture fluids infected with 7 of the 9 virus strains studied. These studies are summarized in table 3. Although infected HeLa tissue culture fluids did not produce strong antigens, they were satifactory and type specific with the standard hyperimmune monkey serum. The use of these antigens with human poliomyelitis convalescent serum has been described previously.[10]

TABLE 3.—*Poliomyelitis-infected HeLa tissue culture fluids as complement-fixing antigens.*

Strain	Type	Harvest time (days)	Antigen titer	Antigen dilution / serum titer
Brunhilde 106	1	2	2	0/256
		3	4	0/256
		4	>4	4/64
		5	>4	4/128
		6	>4	4/64
Pizarro	1	2	>4	0/128
		3	>4	0/128
		4	>4	4/64
		5	>4	2/64
		6	>4	4/64
Y-SK 103	2	2	1	0/32
		3	1	0/64
		4	2	0/256
		5	>4	0/256
		6	>4	2/128
Rodgers	2	2	>4	2/128
		3	>4	2/256
		4	>4	4/16
		5	>4	2/256
		6	>4	4/256
Rrooks	2	2	4	0/128
		3	4	0/128
		4	4	0/256
		5	>4	2/256
		6	>4	2/128
Leon 105	3	2	1	0/4
		3	4	0/256
		4	>4	0/128
		5	>4	0/64
		6	>4	0/128
Eberly	3	2	0	0
		3	0	0
		4	0	0
		5	0	0
		6	0	0
Dolislager	3	2	0	0
		3	0	0
		4	0	0
		5	0	0
		6	0	0
HeLa bottle pools				
Pizarro	1	3	2	0/32
Ender's Br.	1	4	2	0/128
Brunhilde 106	1	4	4	0/256
Brooks	2	5	2	0/128
Leon 105	3	5	8	0/128
Mabie	3	5	8	0/128

10. Miller, C. A. and Baumeister, J. 1955, Antibody responses to naturally occurring poliomyelitis infections in children. 2. Studies by the complement fixation method. Ped. **15**: 392–401.

Antigens derived from dispersed monkey kidney cell cultures.—Poliomyelitis infected fluids of high titer were prepared in cultures of trypsin-dispersed monkey kidney cells, using the technique described by Youngner and Salk[6]. The following studies were devised to determine the complement fixing ability of such fluids.

Cultures were prepared in 200 ml bottles. Twelve ml of a 1/200 dilution (1,000,000 cells per ml) of trypsin-dispersed monkey kidney cells were placed in each bottle. After satisfactory cell growth had occurred (about 7 days), each culture was inoculated with 1.0 ml of virus suspension. Twelve bottles were inoculated with each of three strains of viruses: Ender's Brunhilde, type 1; Brooks, type 2; and Leon, type 3. A different bottle of each strain was harvested every 3 hours for the first 24 hours, and at daily intervals thereafter for 120 hours. Lysis of the cell mass was first observed at the 9th hour and was not complete until the 24th hour. Fluids were used as complement-fixing antigens without any treatment. A box titration of a study with Brunhilde antigen appears in table 1, part C.

TABLE 4.—*Poliomyelitis complement-fixing antigens. Monkey kidney tissue culture fluids.*

Virus strain	Harvest time (hours)	Tissue culture cell degeneration	Antigen titer	Antigen dilution serum titer
Ender's Brunhilde (type 1)	3	0	0	0/0
	6	0	1	0/8
	9	++	1	0/8
	12	++	1	0/4
	15	++	2	0/128
	18	++	2	0/128
	21	+++	2	0/128
	24	+++	8	0/128
	48		8	0/128
	72		8	0/128
	96		8	0/128
	120		4	0/64
Brooks (type II)	3	0	0	0/0
	6	0	0	0/0
	9	++	0	0/3
	12	+++	0	0/0
	15	+++	2	0/64
	18	+++	4	0/64
	21	++++	2	0/64
	24	++++	4	0/64
	48		8	0/128
	72		8	0/256
	96		8	0/256
	120		8	0/256
Leon 105 (type III)	3	0	0	0/0
	6	0	0	0/0
	9	++	0	0/0
	12	++	0	0/0
	15	++	2	0/16
	18	++	4	0/128
	21	+++	8	0/256
	24	+++	4	0/128
	48		8	0/128
	72		16	0/256
	96		8	0/128
	120		8	0/128

Variation in complement-fixing antigenic potency among the three virus strains studied was slight. The results are summarized in table 4. Fluids harvested 15 to 18 hours after inoculation showed significant antigenic activity. This was 6 to 9 hours after the first appearance of cell lysis. Antigenic titers of 1:4 to 1:8 were obtained in 24 to 48 hours when cell lysis was complete, and remained at significant levels through 120 hours. Maximal antigen titers of 1:8 and 1:16 occurred in 24 to 72 hours, with maximal serum titers of 1:128 and 1:256. None of the antigens made from monkey kidney cultures were anticomplementary. They were all type specific with standard monkey serum.

The most potent antigens were prepared by the ultracentrifugation of monkey kidney culture fluids containing poliomyelitis viruses. Fluids were first cleared by cold centrifugation at 10,000 r.p.m. The supernatants were centrifuged at 40,000 r.p.m. in a Spinco vacuum centrifuge (model L) for 1 hour. A small gelatinous pellet was present at the bottom of each cup. These pellets

TABLE 5.—*Summary of results obtained with complement-fixing antigens prepared by ultracentrifugation.*

Type	Antigen titer	Antigen dilution serum titer
Type 1 (Brunhilde)	128	0/64* 16/256
Type 2 (Brooks)	64	0/32 32/512
Type 3 (Leon)	64	0/64 8/256

* A prozone effect in the serum titers was demonstrated when these potent antigens were used in low dilutions.

were resuspended in a small amount of synthetic tissue culture medium. Complement fixation tests using these resuspended pellets as antigens gave the results shown in table 5.

SUMMARY

The poliomyelitis complement-fixing antigenicity of infected tissue culture fluids is presented. Three tissue culture systems were used: monkey testis, HeLa cell, and monkey kidney cultures. The methods of preparing infected fluids are described.

Fluids derived from monkey testis cultures were concentrated by the candle ultrafiltration method. HeLa and monkey kidney culture fluids were unmodified. The most potent antigens were prepared by the ultracentrifugation of fluids from monkey kidney cultures. All methods produced usable antigens. The unmodified antigens had the advantage of being easier to prepare than the concentrated antigens.

Optimal virus growth periods for production of antigens with these infected fluids were shortest for monkey kidney cultures and longest for monkey testis cultures, though other factors, such as differences in the amount of virus in the original inoculations, probably also influenced the optimal harvest time.

LEPTOSPIROSIS CANICOLARIS IN PIGS AND ITS PROBABLE TRANSFER TO HUMAN BEINGS

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In recent years leptospirosis caused by the canicola serotype has been found to occur with strikingly high frequency in both man and animals in Israel. A comparison of recently collected data from this country with the average number of cases in human canicola infections reported from other countries indicates a considerably higher morbidity of this type of infection in Israel (see table 1).

TABLE 1.—*Reported cases of canicola fever in human beings.*

	Average annual number	Population	Number per million reported
Great Britain	17.5	50 million	0.35
The Netherlands	3.3	10 million	0.33
Switzerland	4	5 million	0.8
Israel	12	1½ million	8

The frequent occurrence of *Leptospira canicola* infection in dogs in Israel is evident from table 2, which shows the minimum and maximum figures for the incidence of specific agglutinin reactions for this serotype in dogs, as reported from various countries.

The human cases of canicola fever in this country were diagnosed in our Institute during 31 months, between May 1952 and December 1954, by serological examination of blood samples from persons clinically suspected of leptospirosis. They were mainly forwarded from hospitals in the vicinity of Tel-Aviv. Agglutination in titers of 200 or over were taken to be significant. In most cases further examination revealed a rise of the agglutinin level. As there is ample reason to assume that several human cases of leptospirosis canicolaris are not clinically recognized as such, the real incidence undoubtedly has been still larger than it appears from the official reports or from routine serological examinations.

TABLE 2.—*Percentage of dogs showing significant serotiters for* Lept. canicola.

Germany	1.5–12
The U.S.A.	1.3–38
The Netherlands	20–39
Great Britain	25–40
Israel	20–64 (average 46)

The Israelian data for dogs were collected in different parts of the country by the examination of serums from dogs of the army and the police, dogs of agricultural settlements and stray dogs, during 1953 and 1954.

Until recently, the only known carrier of *Lept. canicola* was the dog, and reports of canicola infection in other animal species are few. In 1952, Yager[1] announced a personal communication by Williams and his associates (see Starr's publication in 1953[2]) concerning 26 persons suffering from canicola fever in Georgia. All of them had contact

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1. Yager, R. H. 1952, Symposium on the Leptispira, Med. Sc. Public., Washington, D. C. pp. 221–222.
2. Starr, L. E. 1953, Proc. Pub. Health Vet. Meeting, Atlanta, Ga., pp. 46–48.

with a swimming pool to which several infected dogs had entrance (64% of the dogs reacted positive for *Lept. canicola*). But also 3 hogs in a pig sty above the pool showed positive agglutination tests with *Lept. canicola*, and a strain of this serotype was recovered from the kidneys of one of them. No gross pathological changes were noted. In Germany Kindler and Schindler[3] obtained positive agglutination with the serum of only one out of 195 pigs. None of 193 pigs examined by Gsell[4] in Switzerland had *Lept. canicola* antibodies in their blood. Van Riel had the same negative results in 260 pigs in Belgium,[5] and in 283 pigs in the Belgain Congo.[6] Covaleda and Pumarola noted negative canicola agglutination tests in 214 pigs in Spain,[7] Schlossberger and Kreuz in 135 pigs in Germany.[8] Whereas 70% of the pig serums in the province of Parma, Italy, reacted with *Leptospira icterohaemorrhagiae*, *Lept. hyos* or *Lept. australis B*, no agglutinins against *Lept. canicola* were recorded by Babudieri and Fiorenti.[9]

In Israel, besides in man and in dogs, leptospirosis canicolaris has now been found to occur in cattle, jackals, and pigs. Moreover, significant serotiters for *Lept. canicola* were encountered in the blood of apparently healthy horses and donkeys on farms where the cattle suffered from this infection. So far we have observed 4 outbreaks of leptospirosis caused by the canicola serotype in cattle.[10] In one settlement where great damage was caused to the lifestock, 59% of 258 calves and heifers showed high agglutinin titers, many of them increasing considerably during the period of observation. *Lept. canicola* was cultivated from a calf with intense jaundice, which died after 2 days illness. In 3 of the 4 places where leptospirosis canicolaris occurred enzootically among cattle, there was no indication that dogs had been the source of infection. However, in the vicinity of these settlements several jackals were found infected by *Lept. canicola*, eliminating the germ in their urine.[11,12] So we came to the conclusion that the jackal plays an important part in the epizootiology of bovine leptospirosis canicolaris in Israel.

The present communication reports two outbreaks of this disease in pigs, both of which were associated with canicola infections in man.

OUTBREAK I

The first observation concerned a herd of more than 200 pigs of different ages, belonging to a pigbreeder in Rishon-Lezion. In June 1954 the entire herd was transferred by truck to a newly erected piggery, situated in a eucalyptus wood on the shore of the Kinnereth Lake near Kfar-Nahum (Kapernaum), a distance of about 200 km from Rishon. This piggery consisted of 22 open concrete-floor sties, separated by approximately 70 cm high concrete walls. The pigs were attended by 8 workers, who, like the pigs lived under very poor hygienic conditions. The sties in which the men usually worked barefooted and with the upper part of their body naked,

3. Kindler, M. and Schindler, R. 1950, Monatschr. prakt. Tierheilk., pp. 14–19.
4. Gsell, O. 1952, Leptospirosen, Bern.
5. van Riel, J. 1952, Bull. Acad. roy. de méd. de Belgique, **17**: 368–383.
6. van Riel, J. and van Riel, M. 1954, Bull. Soc. path. exot. **47**: 435–443.
7. Covaleda, J. and Pumarola, A. 1954, Rev. Ib. Parasitol. **14**: 3.
8. Schlossberger, H. and Kreuz, G. 1954, Ztschr. f. Hyg. **140**: 433–441.
9. Babudieri, B. and Fiorentino, M. 1954, R. C. Ist. Sup. Sanita, **17**: 59–78.
10. van der Hoeden, J. 1955, J. Comp. Path. & Therap. July, **65**: 278–283.
11. van der Hoeden, J. 1955, J. Am. Vet. M. Assoc. **126**: 207–210.
12. van der Hoeden, J. 1955, J. Trop. Med. & Hyg. **58**: 202–204.

were soiled by excretions and food remnants. These wastes drained into the nearby lake, in which the men bathed and from which they took their drinking water. The pigs were returned to Rishon in September 1954.

Human infections.—Between the end of July and the second week of August 1954, i.e., 5 to 6 weeks after the men had started their work, 3 out of the 8 attendants became ill with fever, and they were hospitalized in the Schweitzer Hospital at Tiberias.

Patient S. R., part-time worker, aided in the feeding and cleaning. Fever occurred in the third week of July. The disease took a serious course with anemia, involvement of the liver and kidneys, but without jaundice. The man remained in the hospital for more than a month, intense weakness persisting for an additional 2 or 3 months. The agglutination test, performed on the 23rd day of illness, revealed a high titer (4000) for *Lept. canicola* (table 3).

Patient D. H. became ill at the end of July, clinically suspected of leptospirosis. Agglutinin tests were negative on August 10th, but positive 7 days later (table 3). The course of the disease was moderately serious; no jaundice appeared.

Patient N. B. started working in the piggery in the beginning of July, and became sick in the second week of August. The disease lasted about 2 weeks. Jaundice was not observed. The first agglutinin test, on August 18th, was negative, but on August 26th it had a titer of 16,000 (table 3).

Attendant K. N. came to the piggery at the same day as D. H. Though he never complained of illness, his blood agglutinated *Lept. canicola* in dilution of 1:2000 when tested on August 16th (table 3).

Manager S. E. was present in Kfar-Nahum during the total period of the staying of the pigs in this place, but did not take active part in the care of the animals. Though he remained clinically healthy, his blood agglutinated *Lept. canicola* in dilution of 1:1000 when tested on October 5th.

One of the three remaining workers died from a sudden disease in July 1954. No diagnosis was made. The other two left the place, making examination of their blood on the presence of leptospira antibodies impossible.

Examination of the pigs.—No symptoms of disease were observed in the pigs, either during their stay in Kfar Nahum nor in Rishon-Lezion. On October 30th, blood samples were collected from 33 hogs, selected at random among the pigs which had returned from Kfar-Nahum. Fourteen of these serums showed titers of 200 or more against *Lept. canicola* (titers of 200, 500, 1000 and 2000 were established in 7, 2, 4 and 1 hog, respectively). No agglutinins, however, were revealed for *Lept. icterohaemorrhagiae*, *Lept. pomona* or *Lept. hyos* (*mitis-Johnson*), the serotypes mentioned in the literature of several countries as cause of leptospirosis in swine. Three of the reacting pigs were slaughtered and the kidneys of two of these pigs were examined histologically by Dr. T. Nobel. Diffuse and focal interstitial nephritis and a few evident foci of glomerulonephritis were found, as well as tubular degeneration. In silver-impregnated sections (Levaditti) leptospiras appeared within the epithelium cells of the tubules and in the tubular

TABLE 3.

Attendant	Blood tests, on day of illness	Agglutination titer for leptospira serotype					
		canicola	*ballum*	*icterohaemorrhagiae*	*grippotyphosa*	*pomona*	*hyos*
S.R.	23rd	4000	500	0*	0	0	
D.H.	Approx. 10th	0	0	0	0	0	0
	Approx. 17th	1000	20	0	0	0	0
N.B.	Approx. 8th	0	0	0	0	0	0
	Approx. 16th	16,000	200	0	0	0	0
	Approx. 29th	16,000	500	0	0	0	0
	Approx. 36th	8000	20	0	0	0	0
K.N.	Not ill; tested Aug. 16	2000	0	0	0	0	0
S.E.†	Not ill; tested Oct. 5	1000					

* 0 = less than 20.
† Manager who did not take part in the actual care of the pigs.

lumina. From one of these kidneys a culture of leptospira was isolated in Chang's fluid medium, but, unfortunately, the slowly growing culture was lost before the strain could be typed.

Epidemiology and epizootiology.—The initial negative agglutinin tests of the patients D.H. and N.B., followed by the abundant appearance of *Lept. canicola* agglutinins in their serums at a later stage, proves that the acute disease of these two persons was actually leptospirosis canicolaris. Similarly, the agglutinin titer of 4000 against *Lept. canicola* on the 23rd day of the illness of patient S.R. may be taken as evidence of *Lept. canicola* infection. The fairly high titers of 2000 and 1000 in the healthy persons K.N. and S.E. are in accord with the assumption that they had contracted a subclinical canicola infection in the preceding few months, when exposed to the same heavily contaminated environment as their sick colleagues. A similar condition applied to the clinically inapparent infections of the canicola-seropositive pigs.

The finding of leptospiras in the kidneys of 2 of these pigs may prove that pigs can be a potential source of canicola infection. Though we have no definite certainty that the 5 persons connected with the piggery had acquired their canicola infection from the hogs, we consider this possibility highly probable. Even though the manager, S.E., did not take part in the actual care of the animals, he did have constant close contact with the sties. The only dog present in the vicinity of the piggery at Kfar-Nahum was killed by poison in an anti-rabies campaign against stray dogs and jackals, in July. On two neighboring farms only 3 dogs were present; their blood did not contain agglutinins against *Lept. canicola*. The blood of 22 pigs which were maintained in closed buildings on these farms was also negative. None of the infected men had had any contact with dogs, cattle, horses or donkeys at the time of their stay in the piggery. The origin of the infection of the pigs seemed rather puzzling, though in view of the experiences in cattle, the most probable assumption was to suspect jackals as its source. Unfortunately, local conditions did not enable us to prove that the jackals at this particular place excreted *Lept. canicola* in their urine. Our inclination to attach epizootiological connections between the jackals and the swine does not relate to the period before the latter were transferred to Kfar-Nahum. At Rishon-Lezion the animals were kept in closed buildings into which neither dogs nor jackals could enter, and no contact existed between the pigs and cattle, horses or donkeys.

In the blood serums of 86 pigs from 9 other places (partly in the neighborhood of Rishon), no agglutinins against *Lept. canicola* were found.

OUTBREAK II

This outbreak concerned 35 pigs, approximately 4 months old, living in an open sty outside an abandoned Arab building at the outskirts of Haifa. Inside the house about 200 adult hogs were present. The personnel consisted of the two owners who did not work in the sties, and 4 Arab laborers. At the end of October 1954 the majority of the 35 pigs in the open sty were sick, showing high temperature, anorexia, listlessness, marked weakness and convulsions. Two of the pigs died. Soon after treatment with penicillin and streptomycin was started the temperature dropped.

Examination of the pigs.—On November 3rd, one sick pig which had not been treated was sent to the Veterinary Institute. At arrival the animal was prostrate, with bluish-red, drooping ears and forced respiration. The pig was

destroyed, and post mortem examination showed a large number of petechial hemorrhages in the renal cortex and a swollen spleen. Histological examination (by Dr. T. Nobel) revealed chronic interstitial nephristis, degeneration of the tubular epithelium cells, infiltration of the glomeruli, and fibrous tissue in the interstitium and Bowman's capsule. From the spleen, liver, and blood of the heart pure cultures of *Salmonella suipestifer* were isolated. The blood serum did not agglutinate *S. suipestifer*, but *Lept. canicola* was agglutinated in a dilution of 1:1000 and *Lept ballum* in a dilution of 1:20. The canicola agglutinin titer of the urine was significant (1:32). Attempts to cultivate leptospiras from the kidneys were unsuccessful, since the mediums were quickly overgrown by salmonellas. A young hamster and *Meriones shawi* in which tissue of the kidney was injected died from generalized salmonellosis.

Serum tests of 7 young pigs of the same group revealed one reactor with a canicola titer of 200, one with a titer of 500 and 3 with titers of 1000. The blood of the last 3 animals agglutinated the antigenically related ballum type in dilution 1:20, but none of the 6 reacting serums showed agglutinins for *Lept. icterohaemorrhagiae*, *Lept. pomona* or *Lept. hyos*. Sero-tests with the blood of 6 swine from the adult group were negative.

Human infections.—Three of the four Arabs working in the piggery were ill almost simultaneously, in August and September 1954, after they had been employed from 2 to 4 weeks. During their illness they returned to their families, which lived in the neighborhood of Nazareth. The nature of their disease was not clear to the local physician, who examined them ("malaria, typhoid or influenza?). The main symptoms were: fever for 3 to 4 days and severe headache. Jaundice was not observed. Partial recovery took place in 12 to 18 days but intense weakness remained for several weeks. Only 2 of the Arabs returned to the piggery.

On November 10th, after canicola agglutinins had been found in the pigs, the blood of these 2 workers was tested and agglutinin titers of 1000 and 4000 against *Lept. canicola* were revealed. No agglutinins were present against *Lept. icterohaemorrhagiae*, *Lept. grippotyphosa*, *Lept. pomona*, *Lept. hyos* and *S. suipestifer*. However, *Lept. ballum* was agglutinated in dilution 1:20 and 1:200, respectively.

The blood of the two owners and that of the only worker who remained healthy did not react with *Lept canicola* or any of the other leptospira types mentioned above.

Epidemiology and epizootiology.—No contacts of men or pigs could be traced with dogs, jackals or other possible reservoirs of canicola infection.

SUMMARY AND DISCUSSION

Infections by *Lept. canicola* have been demonstrated in two herds of pigs, one in Galilea near the Kinnereth Sea and the other at the outskirts of Haifa. In the first outbreak no clinical symptoms of disease were observed in the pigs, but at post mortem examination nephritis was revealed. In the second outbreak the young pigs showed rise of temperature, inappetence, marked weakness, convulsions, petechial hemorrhages and nephritis. This outbreak was however, complicated by infection with *S. suipestifer*. The clinical and pathological picture of the latter outbreak showed a striking resemblance to the outbreak described by Gochenour et al[13] in which the pigs were found to be infected by *S. suipestifer*, besides

13. Gochenour, W. S., Jr., Johnston, R. V., Yager, R. H. and Gochenour, W. S. 1952, Am. J. Vet. Res. pp. 158–160.

showing significant agglutinin titers against *Lept. pomona*.

Though a very slight possiblity cannot be denied that a dog had been the original source of infection in the Kfar-Nahum outbreak, we consider it more likely that the contamination of the swine derived from jackals. These animals could easily enter the sties at night in search of food, depositing *Lept. canicola* by their urine on the soil. In previous investigations in Israel, jackals have been found to be excretors of this leptospira serotype.[11] In the Haifa outbreak the possible origin of infection could not be traced.

Both outbreaks of leptospirosis among pigs were associated with multiple human infection (5 and 3 cases, respectively). These data constitute a striking contrast to the great exception by which, according to general experience, leptospirosis is usually transferred from the excreting dogs to man. To quote a few examples: In Austria only 2 human cases of leptospirosis were diagnosed among the owners of 7370 leptospira-infected dogs, and examination of the personnel handling these dogs, in the Veterinary Clinics at Vienna, revealed not a single case of leptospirosis.[14] Among the 33 cases of canicola fever which we observed in Israel and in which dogs were the probable source of infection, it happened only once that 2 persons in the same house were affected.

A reason for the occurrence of multiple human infection connected with the outbreaks of porcine leptospirosis described above may probably be sought in the very unhygienic conditions of the pigsties. Bathing in and drinking water from a lake into which the piggery drained may have been an additional way of infection of the human cases connected with the infected hogs at Kfar-Nahum.

Irrespective of whether the *Lept. canicola* infections in human beings were derived from jackals or via pigs, we believe that the observations reported above and those which have been described in our previous publications[10,12] necessitate reconsideration of the general conception that canicola fever is a domestic rather than an occupational disease. In some countries, under certain conditions at least, it might well be the other way round.

14. Advances in the Control of Zoonoses, FAO-Public. Agric. Studies, no. 25, 1953, p. 153.

SOME EFFECTS OF CORTISONE IN MICE WITH ACQUIRED IMMUNITY TO *TRICHINELLA SPIRALIS*

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In a previous report (Coker, 1955) it was demonstrated that treatment of nonimmunized, mature mice with cortisone lowered immunity against a primary infection with *Trichinella spiralis* larvae. In the cortisone-treated mice, there was a longer persistence of more adult worms in the intestine and the establishment of much larger numbers of larval worms in the musculature than in control mice. These results supported the view that nonimmunized mice develop, within a relatively brief time after a primary infection, a degree of acquired immunity against this nematode.

Because cortisone exerted such a striking effect on the acquisition of immunity to a primary infection in mice, it was decided to test whether this hormone would similarly break down the stronger immunity acquired by mice to repeated infections with *T. spiralis*. From a survey of the literature it was to be expected that cortisone probably would be effective in reducing the cellular response in the intestine, which was shown by Larsh and Race (1954) to be associated with immunity to *T. spiralis*. If so, the information obtained from its use might be of value in understanding the mechanism(s) of this immunity. The current experiment measures the effects of various dosages of cortisone upon the persistence of adult worms in immunized mice following a challenging infection, and includes certain rough measurements (spleen size and qualitative blood cell counts) of the effects of these same dosages of the hormone upon the lymphoid-macrophage system. A third, final experiment of this series which has been reported so far only in the original dissertation (Coker, 1954) presents the effect(s) of cortisone upon the cellular response in the intestine and its relation to the operation of immunity. This work will be reported in a later paper.

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MATERIALS AND METHODS

The source of mice and of *T. spiralis* larvae, the general methods involved in infecting the animals, making adult worm counts, preparing and injecting the cortisone, and analyzing the data statistically were as indicated previously (Coker, 1955).

EXPERIMENTAL PROCEDURES AND RESULTS

Experimental design.—The purpose of the experiment was threefold to determine (1) whether cortisone would have effects upon immunity established against *T. spiralis* in mice as measured by adult worm burdens in the small intestine following a challenging infection, (2) whether cortisone would have effects upon the lymphoid-macrophage system of these same mice as judged by

spleen sizes and differential blood cell counts, and (3) whether any of the observed effects of cortisone would be dependent upon the amount of the hormone injected.

For the satisfaction of these aims, the experiment was designed as follows. Seventy male mice 8 weeks old were selected. Ten were kept as nonimmune controls, and 60 were immunized by giving them, at 3-week intervals, three separate infections with 200 *T. spiralis* larvae. Proof for the infectivity of the larvae used in these immunizing infections was provided by including additional "viability controls" killed 7 days after infection for counts of adult worms. Twenty-seven days after the last stimulating infection was given, the 60 immunized and the 10 nonimmunized mice were divided into 7 main groups (I to VII) of 10 mice each (table 1). The cortisone experimentals (groups II to V) were given, by the subcutaneous route, their first dose of cortisone on this day; the hormone was given daily thereafter until necropsy. Three days later, or 30 days after the last stimulating infection was given to the immunized mice, all of the mice, except the 10 mice of group I (see below), received a challenging infection with 400 *T. spiralis* larvae. At this point, each of the 7 main groups of mice was divided into two subgroups, (a) and (b), of 5 mice each, which were sacrified for counts of adult worms in the small intestine, for spleen size measurements and for differential blood cell counts at 7 days (a) and 14 days (b) following the challenging infection. The spleens were measured fresh for maximum length and width in millimeters. Differential blood cell counts were made from clipped tail blood smears stained with Wright's stain. Lymphocytes, neutrophils, monocytes and eosinophils were differentiated. Eosinophils did not present the characteristic appearance expected from examining other types of blood stained by the conventional techniques; this observation agrees with that of Dunn (1954) in another strain of mice. To clarify the design, a brief description of the various groups of mice (table 1) follows.

Group I (persistency controls) was

TABLE 1.—*Average adult worm counts, spleen sizes and differential blood counts in various groups of mice 7 and 14 days following a challenging infection with 400* T. spiralis *larvae given to groups II to VII. Groups I to VI were immunized prior to challenge.*

		Day	I Persistency controls (10 mice)	Cortisone experimentals II 0.25 mg (10 mice)	III 0.5 mg (10 mice)	IV 1.0 mg (10 mice)	V 2.0 mg (9 mice)	VI Immune controls (10 mice)	VII Nonimmune controls (10 mice)
Adult worm counts		7	0.2	310.8	329.8	325.2	331.8	254.4	347.6
				324.4					
		14	0.2	276.0	317.4	285.6	288.5	49.8	158.4
				292.1					
Spleen size (mm)			L* W*	L W	L W	L W	L W	L W	L W
		7	15.2 4.1	12.7 3.7	12.0 3.2	12.4 3.4	11.8 3.1	15.0 4.1	17.0 4.7
		14	14.9 4.3	12.5 3.3	12.4 3.2	12.0 3.1	12.4 3.1	15.7 4.4	14.8 3.9
Percent white cells	L†	7	54.0	14.4	12.6	2.4	1.8	35.6	46.6
	N		23.6	69.4	78.8	92.6	97.4	48.0	42.4
	M		6.4	8.2	4.8	4.6	0.2	5.2	6.4
	E		16.0	8.0	3.8	0.4	0.6	11.2	4.6
	L	14	45.8	20.6	11.4	2.6	1.0	44.2	41.6
	N		27.2	64.6	75.8	95.6	98.5	26.0	47.2
	M		8.6	5.6	5.0	1.8	0.5	6.0	3.2
	E		18.4	9.2	7.8	0.0	0.0	23.8	8.0

* L, length, W, width.
† L, lymphocyte, N, neutropil, M, monocyte, E, eosinophil.

immunized but not challenged, its function being to determine if worms persisted from the last immunizing infection to complicate the worm counts of the challenging infection. Very few worms were found to have persisted, but the results from the persistency group are included because of their bearing upon spleen measurements and differential blood cell counts.

Groups II, III, IV and V (cortisone experiments) were immunized, challenged and treated with cortisone in daily doses of 0.25, 0.5, 1.0 and 2.0 mg, respectively. The daily dose most frequently used in mice for various purposes by other workers has been 1 mg. Thus, these four dosages, selected arbitrarily, were one-fourth, one-half, equal and twice that amount. It is well to emphasize that in the present design the hormone was first given 3 days prior to the challenging infection and continued until necropsy.

Group VI (immune controls) was immunized and challenged, to establish the base-line of acquired immunity as compared to group VII (nonimmune controls), which was not immunized but was challenged to show the degree of natural immunity in mice of this age.

The 7-day and 14-day periods for necropsy after the challenging infection were selected on the basis of previous observations (Larsh et al, 1952) showing that after a challenging infection immunized mice of this same strain lost their worms in significant numbers between 5 to 7 days, whereas nonimmunized mice lost theirs between 11 to 14 days. Since the effect of cortisone on the established immunity was unknown a priori, the use of the two necropsy periods was expected to permit more comparisons leading to better evaluation of the results.

General results.—All mice survived to necropsy except one mouse in group V (b) which was killed accidentally. The cortisone-treated animals appeared to be as active as the others but lost some weight. Averaged data for the adult worm counts, the spleen size measurements and the differential blood cell counts are shown in table 1. Results of *t* tests of the significances of the differences between the means of the adult worm counts are given in table 2; a probability of 0.05 was accepted as significant.

Adult worm counts.—The average worm counts in the groups treated with 0.25, 0.5, 1.0 and 2.0 mg of cortisone were 310.8, 329.8, 325.2 and 331.8 at 7 days, and 276.0, 317.4, 285.6 and 288.5 at 14 days, respectively. An analysis of variance performed on these counts indicated that there were no differences to be ascribed to the size of dose of cortisone. Therefore, for further calculations the cortisone groups could be combined, which resulted in a mean of 324.4 worms

TABLE 2.—T *tests of the significances of the differences between the means of adult worm counts.*

Comparisons		Standard Error ($\bar{X}_1 - \bar{X}_2$)	*t* value	Significance level
No.	Type			
1	$Immune_7$ vs. $nonimmune_7$	44.1	2.11	0.05
2	$Immune_{14}$ vs. $nonimmune_{14}$	44.1	2.46	0.02
3	$Immune_7$ vs. $immune_{14}$	44.1	4.64	<0.01
4	$Nonimmune_7$ vs. $nonimmune_{14}$	44.1	4.29	<0.01
5	$Immune_7$ vs. $cortisone_7$	35.0	2.00	0.05
6	$Cortisone_7$ vs. $nonimmune_7$	35.0	0.66	NS
7	$Immune_{14}$ vs. $cortisone_{14}$	35.0	7.12	<0.01
8	$Cortisone_{14}$ vs. $nonimmune_{14}$	35.0	3.82	<0.01
9	$Cortisone_7$ vs. $cortisone_{14}$	22.3	1.45	0.10

Standard errors of the differences between the means were calculated using the pooled variances of all the groups, giving 53 degrees of freedom.

at 7 days and a mean of 292.1 worms at 14 days. The mean worm counts of the persistency controls, the immune controls and the nonimmune controls were 0.2, 254.4 and 347.6 at 7 days and 0.2, 49.8 and 158.4 at 14 days, respectively.

Following the use of Student's t test to determine the significance of the differences between the means of the various adult worm counts (table 2), the following conclusions were made with confidence: (a) The immunization program was successful in establishing immunity, detectable at both 7 and 14 days in the immune controls (comparisons 1 and 2 in table 2); (b) the immunity mechanism continued to operate between 7 and 14 days in the immune controls (comparison 3); (c) nonimmune controls gained detectable acquired immunity between 7 and 14 days (comparison 4); (d) cortisone-treated mice at 7 days had significantly more worms than immune controls (comparison 5), and their worm burdens were not significantly different from those of the nonimmune controls (comparison 6); (e) at 14 days the cortisone-treated mice harbored significantly more worms than both the immune controls (comparison 7 and the nonimmune controls (comparison 8); and (f) worm burdens of the cortisone-treated mice at 14 days probably were not significantly different from their burdens at 7 days (comparison 9).

Spleen size.—The range of average spleen lengths in the cortisone groups was 11.8 to 12.7 mm, with a mean of 12.3 mm, and that of widths was 3.1 to 3.7 mm, with a mean of 3.3 mm, as opposed to 14.8 to 17.0 mm, with a mean of 15.4 mm, and 3.9 to 4.7 mm, with a mean of 4.3 mm, respectively, for all the other groups not receiving cortisone (table 1). Thus, the 39 mice of the cortisone groups had a spleen size which averaged 3.1 mm shorter in length and 1.0 mm shorter in the width than that of the 30 mice of the other groups. There was little difference in average spleen size within the cortisone groups irrespective of necropsy time, indicating that cortisone induced prompt and maximal reduction of spleen size.

Differential blood cell counts.—A casual inspection of the differential blood cell counts from group to group (table 1) indicates: (a) an eosinophilia developed in non-cortisone-treated immune groups I and VI; (b) a lymphocytopenia and eosinopenia occurred in the cortisone-treated groups II to V; and (c) the effects of cortisone upon lymphocytes and eosinophils were greater as the dosage of cortisone increased. In making the blood cell counts much more time was required to find 100 white cells in the smears from the cortisone-treated animals. Therefore, although these counts are not quantitative measures of the total amount of various leucocytes present, it is reasonably safe to assume that cortisone reduced the population of circulating lymphocytes and eosinophils to very low levels. This is in accordance with the known action of cortisone reported by other workers who have made total white cell determinations (see below).

DISCUSSION

The results of this experiment show clearly that cortisone, as employed here, drastically affected the maintenance of immunity that had been established in mice by repeated infections. The effect was to destroy the established immunity as measured by the numbers of adult worms persisting in the small intestine after a challenging infection. Cortisone treatment rendered immunized mice essentially nonimmune and, in addition, seemed to prevent the gain of acquired immunity which nonimmunized mice ordinarily develop between 11 to 14 days following an infection (Larsh, 1953; Larsh and Race,

1954). The results of this experiment with immunized mice substantiate earlier findings of the effect of cortisone in nonimmunized mice infected with *T. spiralis*.

Cortisone also had a demonstrable effect upon the lymphoid-macrophage system of the immunized mice in that it caused a reduction in size of the spleen, a lymphocytopenia and an eosinopenia. Similar effects of cortisone or ACTH have been noted in animals not infected with *T. spiralis* (Dougherty and White, 1945 and 1947; Hills et al, 1948; Antopol, 1950).

The various doses of cortisone produced no significant differences among themsleves in the effect upon adult worm counts or upon size of the spleen. However, higher doses affected differential blood cell counts to greater degree, although there was not much difference in this respect between the 0.25 mg and the 0.5 mg doses. The matter of selection of an appropriate dose of cortisone for various experimental treatments of mice has not received a great deal of attention, but two references pertinent to the present study deserve mention. Stoner and Godwin (1953), in one experiment with cortisone and trichinosis in mice, tested 1.0 mg and 0.1 mg of cortisone, administered as daily subcutaneous injections, for their effects upon an LD_{50} of larvae. Although the challenging infection of *Trichinella* produced 100% mortality, a criterion of survival time placed the minimal effective dosage somewhere between 0.1 and 1.0 mg of cortisone. Spain and Molomut (1952) found that a dosage of as little as 0.00025 mg produced pronounced eosinopenia in mice, whereas the effect upon spleen size began at 0.025 mg and was maximal at 0.25, 1.0, 2.0 and 4.0 mg. In the current experiment, the threshold level of effects on adult worm counts was not demarked, but it would seem to lie below 0.25 mg. It is well to bear in mind, however, that the strain of mouse used (Whitmire and Downs, 1954), as well as the size of cortisone dose employed, can be an important additional factor to consider when measuring the effects of cortisone.

The influence of cortisone on normal host immunity to disease varies with the disease (Kass and Finland, 1951; Greiner, 1952), but in the case of infectious diseases the influence often is adverse. In mice, for example, it has caused a marked acceleration of poliomyelitis infection (Shwartzman, 1950); an endogenous bacteremia (Berlin et al, 1952); a generalized fatal infection with *Candida albicans* (Seligmann, 1953); greater susceptibility to *Rickettsia mooseri* (Whitmire and Downs, 1954); and enhanced susceptibility to *Trichinella* (Stoner and Godwin, 1953 and 1954; Coker, 1954 and 1955).

Thomas (1953) pointed out that enhancement of susceptibility produced by cortisone involves a wide variety of unrelated infectious agents and a wide variety of hosts. He suggested that the underlying mechanism by which cortisone exerts such effects is through blockade of the reticuloendothelial system, thus preventing cellular defenses from coming into play. Evidence is accumulating that this explanation may apply in at least certain cases of nematode infections. Weinstein (1953 and 1955) found that cortisone administered to rats during or after immunization to *Nippostrongylus muris* caused a marked reduction in the cellular response in the skin to a challenging infection with larval worms, and the worms were more successful in continuing their migration and development although they encountered some cellular resistance in the internal organs. Coker (1954) has shown that there is a direct relationship between suppression of the cellular response in the intestine

and the persistence of large numbers of adult *T. spiralis* in mice treated with this hormone.

SUMMARY

1. Cortisone treatment of mice immunized against *Trichinella spiralis* caused longer persistence of adult worms in the small intestine after a challenging infection, reduction in size of the spleens of the mice, lymphocytopenia and eosinopenia.

2. The various daily dosages of the hormone (0.25, 0.5, 1.0, and 2.0 mg) produced no significant differences among themselves in their effects upon adult worm counts or upon size of the spleen. However, dosages above 0.5 mg affected differential blood cell counts to greater degree.

3. Cortisone treatment rendered the immunized mice essentially nonimmune, and, in addition, seemed to prevent in these mice the gain of acquired immunity that nonimmunized mice ordinarily develop between 11 to 14 days following *T. spiralis* infection.

4. The results of this experiment with immunized mice substantiate earlier findings of the effect of cortisone in nonimmunized mice infected with *T. spiralis*.

REFERENCES

Antopol, W. 1950, Anatomic changes produced in mice treated with excessive doses of cortisone. Proc. Soc. Exper. Biol. & Med. **73**: 262–265.

Berlin, B. S., Johnson, C., Hawk, W. D. and Lawrence, A. G. 1952, The occurrence of bacteremia and death in cortisone-treated mice. J. Lab. & Clin. Med. **40**: 82–89.

Coker, C. M. 1954, Effects of cortisone on established immunity to *Trichinella spiralis* in mice, with reference to the role of the cellular factor(s) in the immunity mechanism. Doctoral thesis, University of North Carolina, Chapel Hill, N. C.

———. 1955, Effects of cortisone on *Trichinella spiralis* infections in non-immunized mice. J. Parasitol. In press.

Dougherty, T. F. and White, A. 1947, Functional alterations in lymphoid tissue by pituitary-adrenal cortical secretion. Am. J. Anat. **77**: 81–116.

Dunn, T. B. 1954, Normal and pathologic anatomy of reticular tissue in laboratory mice. J. Nat. Cancer Inst. **14**: 1281–1434.

Greiner, T. 1952, The current uses of cortisone and ACTH. Am. J. Sc. **223**: 553–568.

Hills, A. G., Forsham, P. H. and Finch, C. A. 1948, Changes in circulating leucocytes induced by the administration of pituitary adrenocorticotropic hormone (ACTH) in man. Blood **3**: 755–768.

Kass, E. H. and Finland, M. 1951, The role of adrenal steroids in infection and immunity. New England J. Med. **244**: 464–470.

Larsh, J. E., Jr. 1953, Studies in old mice to test the hypotheses of local and general immunity to *Trichinella spiralis*. J. Infect. Dis. **93**: 282–293.

Larsh, J. E., Jr., Gilchrist, H. B. and Greenberg, B. G. 1952, A study of the distribution and longevity of adult *Trichinella spiralis* in immunized and non-immunized mice. J. Elisha Mitchell Sc. Soc. **68**: 1–11.

Larsh, J. E., Jr. and Race, G. J. 1954, A histopathologic study of the anterior small intestine of immunized and nonimmunized mice infected with *Trichinella spiralis*. J. Infect. Dis. **94**: 262–272.

Seligmann, E. 1953, Virulence enhancement of *Candida albicans* by antibiotics and cortisone. Proc. Soc. Exper. Biol. & Med. **83**: 778–781.

Shwartzmann, G. 1950, Enhancing effect of cortisone upon poliomyelitis infection (strain MEF[1]) in hamsters and mice. Proc. Soc. Exper. Biol. & Med. **75**: 835–838.

Spain, D. M. and Molomut, N. 1952, Effect of cortisone on inflammation in mice. Am. J. Clin. Path. **22**: 944–947.

Stoner, R. D. and Godwin, J. T. 1953, The effects of ACTH and cortisone upon susceptibility to trichinosis in mice. Am. J. Path. **29**: 943–950.

Stoner, R. D. and Godwin, J. T. 1954, The effects of adrenocorticotrophic hormone and cortisone upon acquired immunity to trichinosis in mice. Am. J. Path. **30**: 913–918.

Thomas, L. 1953, Cortisone and infection. Ann. N. Y. Acad. Sc. **56**: 799–814.

Weinstein, P. P. 1953, The effect of cortisone on the development of the immune response in the white rat to *Nippostrongylus muris*. J. Parasitol. (Aug. suppl.) **39**: 35.

———. 1955, The effect of cortisone on the immune response of the white rat to *Nippostrongylus muris*. Am. J. Trop. Med. & Hyg. **4**: 61–74.

Whitmire, C. E. and Downs, C. M. 1954, Effect of cortisone on experimental murine typhus in mice. Science, **119**: 468–469.

ISOLATION OF SIX STRAINS OF ORNITHOSIS VIRUS FROM CHILDREN WITH INFECTIONS OF THE RESPIRATORY TRACT

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Little is known of psittacosis or ornithosis in very young children,[1] the majority of recorded cases having been adults.[1–11]

A virus belonging to the ornithosis group was isolated in this laboratory in 1953 from two fatal cases of interstitial pneumonia in children aged 2 years and 1 month,[12] respectively, and more cases in young children were detected in 1954. The present paper deals with virological and serological findings in these patients and some of their epidemiological implications. A clinical description has been reported elsewhere.[13]

METHODS

Virus isolation.—Virus isolation was attempted from patients' serums or whole blood at the beginning of the illness (sputum was not obtainable because of the young age of the children), or from lung and spleen obtained at autopsy. As far as possible material was inoculated immediately on receipt; otherwise it was stored at −20 C for not more than a few days. For inoculation blood or serum was diluted with the same volume of nutrient broth containing 50 units of penicillin and 50 μg of streptomycin/ml. Portions of lung and spleen were prepared by trituration in a sterile mortar, and broth containing 200 units of penicillin and 200 μg of streptomycin/ml was added to make a 20% suspension.

Twelve- to 13-day-old developing chick embryos were inoculated amniotically with these materials. After incubation at 36 C for 4 to 5 days, amniotic fluid, liver and lungs of the embryos were removed, pooled and triturated to make a 20% suspension in broth. Further passages in eggs were done by the amniotic and yolk

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1. Strobel, W. 1954, Beitrag zum Krankheitsbild der Ornithose im Kindesalter. Deutsche med. Wchnschr. **79**: 176–180.
2. Bedson, S. P. 1950, Primary atypical pneumonia. Brit. M. J. **II**: 1461.
3. Eator, M. D., Beck, M. D. and Pearson, H. E. 1941, Virus from cases of atypical pneumonia; relation to viruses of meningopneumonitis and psittacosis. J. Exper. Med. **73**: 641–654.
4. deGara, P. F. and Furth, J. 1948, Pneumonia produced by a meningopneumotropic virus. Report of a fatal case, with observations on the interrelationship of psittacosis-like viruses. Arch. Path. **45**: 474–493.
5. Meyer, K. F. and Eddie, B. 1951, Human carrier of the psittacosis virus. J. Infect. Dis. **88**: 109–125.
6. Meyer, K. F. and Eddie, B. 1947, The knowledge of human virus infections of animal origin. J.A.M.A. **133**: 822–828.
7. Meyer, K. F. and Eddie, B. 1942, Spontaneous ornithosis (psittacosis) in chickens the cause of a human infection. Proc. Soc. Exper. Biol. & Med. **49**: 522–525.
8. Olson, B. J. and Treuting, W. L. **1944**, An epidemic of a severe pneumonitis in the Bayou region of Louisiana. Pub. Health Rep. **59**: 1299–1311.
9. Siegel, M. M., Cole, L. S. and Hunter, O. 1953, Mounting incidence of psittacosis. Am. J. Pub. Health, **43**: 1418–1422.
10. Smadel, J. E. 1943, Atypical pneumonia and psittacosis. J. Clin. Investigation, **22**: 57–65.
11. Zichis, J. and Shaughnessy, H. J. 1945, Isolation of an apparently new virus from two fatal pneumonia cases. Science, **102**: 301–332.
12. Ephrati-Elizur, E., Bernkopf, H. and Wolman, M. 1953, Isolation of a virus belonging to the psittacosis-lymphogranuloma group from two fatal cases of interstitial pneumonia in children. Harefuah, **45**: 199–203.
13. Berman, S., Freundlich, E., Glaser, K., Ephrati-Elizur, E. and Bernkopf, H. 1955, Ornithosis in early childhood. Pediatrics, **15**: 752–760.

sac route. Chorioallantoic inoculation was carried out by conventional methods.

Three- to 4-week-old albino mice were inoculated intracerebrally and intranasally with egg passage material after amniotic inoculation, or directly with serum, blood or lung and spleen suspensions from human cases. Mice inoculated intranasally were sacrificed after 7 to 10 days and the lungs removed for macroscopic and microscopic examination. Mice inoculated intracerebrally were observed for signs of illness and death. Lung and brain smears were stained by the Macchiavello and Giemsa methods for the detection of elementary and inclusion bodies.

Normal mice from our stock were tested repeatedly for latent infection with pneumotropic viruses by inoculating them intranasally with normal amniotic fluid, suspensions of chick embryo organs, lung suspensions of normal mice and normal human serum. A number of blind passages were carried out, using a total number of over 200 mice. No deaths occurred. Animals were sacrificed 7 to 10 days after inoculation and examined for pathological changes. None was found, indicating that the stock of mice was free from latent respiratory infection.

Hamsters, guinea pigs and pigeons were also inoculated by various routes in the investigation of biological characteristics of the viruses isolated.

Serological examinations.—Acute and convalescent serums from cases of respiratory diseases were examined for complement fixing (CF) and hemagglutination inhibiting (HI) antibodies against psittacosis antigen. CF antigens were prepared from allantoic fluid of embryonated eggs infected with strain 38, which had been isolated from a fatal case of pneumonia and identified as a strain belonging to the ornithosis group. Allantoic fluid from infected eggs found to be rich in elementary bodies on microscopic examination was dialyzed overnight against 20 volumes of saline at 4 C. To the dialysate merthiolate in a final dilution of 1:10,000 was added. Control antigen was prepared in the same way from normal allantoic fluid. Specific antigens reacted up to a dilution of 1:4 with 4 units of immune serum. Two units of antigen were used in the test. Serums were inactivated for 30 minutes at 56 C.

Fresh guinea pig serum was used for complement and unbuffered saline as diluent. The tests were carried out in a total volume of 1 ml, using 0.2 ml volumes of each reagent, 2 units of complement and 2 units of amboceptor.

Preliminary incubation was carried out at 4 C overnight followed by 15 minutes at room temperature. The hemolytic system was then added and further incubation carried out in a water bath at 37 C for 30 minutes. Titration of complement in the presence of the infected and the control antigen and all other controls were run together with the test. Tests were only considered satisfactory when 2 units of complement were present in the control titrations in the presence of antigen after overnight incubation and the result considered positive when 2 to 4+ fixation was obtained.

Hemagglutination inhibition tests were done according to the technique of Hilleman et al,[14] preliminary absorption of the human serums with mouse red cell suspensions was carried out. Allantoic fluids exhibiting hemagglutination activity in titers of 1:32 or higher were employed as antigens. Four hemagglutination units were used in the inhibition test.

All titers are reported as the highest initial serum dilution causing complete or partial hemagglutination inhibition or complement fixation.

Preparation of viral toxins were carried out according to the technique proposed by Manire and Meyer.[15]

RESULTS

Virus isolation was attempted in 24 cases of respiratory disease in children. These included 18 cases from which blood was received at the beginning or later stages of the disease, and 6 fatal cases of pneumonia from which lung and spleen were obtained at autopsy.

Isolation was successful on 6 occasions. Three virus strains were isolated from blood samples and three strains from autopsy material (table 1).

For virus isolation eggs were inoculated by the amniotic route. This method was chosen in the first positive case because infection with influenza virus was originally suspected and it was subsequently adopted in all further isolation attempts. In 4 cases

14. Hilleman, M. R., Haig, D. A. and Helmond, R. J. 1950, The indirect complement fixation, hemagglutination and conglutinating complement absorption tests for viruses of the psittacosis-lymphogranuloma venereum group. J. Immunol. **66**: 115–130.
15. Manire, G. P. and Meyer, K. F. 1950, The toxins of the psittacosis-lymphogranuloma group of agents. J. Infect. Dis. **86**: 226.

TABLE 1.—*Cases of respiratory disease from which virus strains were isolated.*

Case no.	Age	Virus isolation on day of illness	Material for isolation	Outcome	Clinical diagnosis or autopsy findings
38	2 years	21	Lung and spleen	Fatal	Interstitial pneumonia
62	1 month	1 (sudden death)	Lung and spleen	Fatal	Hemorrhages found in lungs at autopsy
898	1½ months	5	Serum	Recovered	Upper respiratory infection
900	2 years	3	Serum	Recovered	Upper respiratory infection
996	1 month	14	Blood	Recovered	Pneumonia
1050	11 months	37	Blood	Fatal	Bronchopneumonia

virus was first demonstrated microscopically in touch preparations of mouse lung following intranasal inoculation with amniotic fluid. In two further cases amniotic fluid passaged to the yolk sac caused death of the embryos after 4 to 5 days, and virus was directly demonstrated in yolk sac preparations (table 2).

Identification of the viruses isolated.—Impression preparations made from lungs and brains of infected mice, and smears from allantoic fluid or yolk sac of inoculated embryonated eggs stained by the Macchiavello or Giemsa method, showed elementary and inclusion bodies typical of the psittacosis-lymphogranuloma group.

For further classification of the isolated strains a number of factors were taken into account, such as: source of isolated strains, virulence in mice following intraperitoneal and intracerebral injection, virulence in pigeons following intracerebral inoculation, neutralization tests, and cross immunity tests.

Pathogenicity of isolated strains.—The pathogenicity of all isolated strains for developing chick embryos, mice and pigeons was examined. Developing chick embryos were susceptible to inoculation into the yolk and amniotic sac and into the allantoic cavity. Death of the embryos occurred 3 to 6 days after infection, depending on the route. Following amniotic inoculation, virus was found not only in the amniotic fluid, but also in the liver and lungs of the embryo, reaching titers of $10^{7.6}$/ml. Chorioallantoic inoculation produced small focal lesions on the membrane.

Mice died within 3 to 6 days following intracerebral inoculation. Mouse brain suspensions reached titers of 10^7 to 10^8 /ml after 6 passages. Intranasal inoculation was not usually fatal to mice, but those sacrificed after 7 to 10 days showed typical greyish lesions in their lungs, which varied from pin point size to almost complete consolidation, depending on the amount of virus inoculated. Mice inoculated by the intraperitoneal route seldom died. In animals sacrificed 10 days after intraperitoneal inoculation, livers and

TABLE 2.—*Methods used for virus isolation.*

Case no.	Material	Number of passages in:	
		Eggs	Mice
38 & 62	Lung and spleen suspension	1. Amniotic inoculation 2. Amniotic inoculation 3. Amniotic inoculation 4. Chorioallantoic inoculation confluent membrane lesions	i.c.—negative i.c.—negative i.n.—lungs infected i.c.—death in 3 days
898 & 900	Serum	1. Amniotic inoculation	i.n.—typical lung lesions after 7 days i.c.—death in 3 days
996 & 1050	Whole blood	1. Amniotic inoculation 2. Yolk sac inoculation—death of embryo. Elementary bodies demonstrated	i.n.—typical lesions i.c.—death in 5–6 days

i.n. = intranasal inoculation, i.c. = intracerebral inoculation.

spleens were found to be considerably enlarged. Microscopic examination of smears from liver, lungs and spleen stained by the Giemsa stain showed elementary and inclusion bodies.

White rats and hamsters inoculated intracerebrally with 10% mouse lung suspension (strain 38) succumbed within 5 to 7 days. Guinea pigs survived all routes of inoculation. Injection of infected yolk sac suspension intracutaneously in guinea pigs was followed by a strong inflammatory local reaction after 48 hours. Intracerebral inoculation of pigeons with infected yolk sac suspensions of all 6 strains produced signs of illness within 3 to 5 days and death 4 to 5 days later. Brain smears revealed numerous elementary bodies and mice inoculated intracerebrally with pigeon brain suspensions succumbed to the infection within 3 days.

Toxin production.—Toxin production is one of the characteristics of viruses of the psittacosis—LGV group. Differences in toxin production between various members have been described.[15] It was of interest, therefore, to examine the isolated strains in this respect.

Three of the strains isolated and one strain of ornithosis virus were studied for the presence of viral toxins. Yolk sac suspensions in dilutions up to 1:40, inoculated by the intravenous route, killed all or almost all mice within 40 hours. Animals surviving 40 hours did not die from the injection later. All the strains tested behaved similarly.

Cross protection test in mice.—Three groups of 20 to 50 mice each were immunized by a single subcutaneous injection of 0.5 ml of fresh allantoic fluid containing numerous elementary bodies diluted 1:10 in broth. The strains used for immunization were: (1) a local ornithosis strain isolated from sick pigeons, (2) strain 38, (3) strain 900; a group of 75 mice was kept as control animals. Three weeks later all mice were challenged intracerebrally with 10^4 LD_{50}'s of homologous or heterologous strains. As seen in table 3 all immunized mice survived the challenge with both homologous and heterologous strains, while all the animals in the nonimmunized group succumbed.

TABLE 3.—*Results of cross protection tests in mice.*

Immunization with strain	Intracerebral challenge with strain			
	Ornithosis	38	898	900
Ornithosis	0/20	0/20		0/10
38	0/20	0/20	0/13	
900	0/10			0/10
Control (Not immunized)	20/20	20/20	13/13	10/10

Numerator—number of total infections, denominator—number of mice inoculated.

These results indicate the close immunological relationship of the strains examined, both among themselves and with ornithosis virus.

Chickens were immunized by repeated intrasmuscular inoculations of infected yolk sac suspensions. No potent neutralizing antibodies were obtained and efforts to carry out serum neutralization tests were therefore abandoned.

Sensitivity to antibiotics.— It is known that some members of the psittacosis-LGV group are sensitive to sulfonamides and antibiotics. Sensitivity to antibiotics of three of the isolated strains and of ornithosis virus was tested according to the technique described by Meiklejohn et al.[16] There was no difference in survival time between eggs pretreated with 10 mg of sodium sulfadiazine or 100 units of penicillin and untreated control eggs. Aureomycin in a dose of 1 mg per egg completely inhibited viral multiplication, as shown by the survival of all inoculated embryos, whereas 0.1 mg aureomycin or 1000

16. Meiklejohn, G., Wagner, J. C. and Beveridge, G. W. 1946, Studies on the chemotherapy of viruses in the psittacosis-lymphogranuloma group. J. Immunol. **54**: 1.

TABLE 4.—*Serological reactions in cases of atypical pneumonia.*

Case no	Age	Sample I: Days of illness	Sample I: CF	Sample I: HI	Sample II: Days of illness	Sample II: CF	Sample II: HI	Virus isolated	Parent's serum tested
737	2 years	8	1:16++ −+++	1:160	*			Not attempted	Not available
784	5 months	12	1:8 neg.	1:160	*			Not attempted	Not available
553	6½ months	14	1:8 neg.		32	1:8++ −+++	—	Not attempted	Not available
561	9 months	16	1:16++++	1:320	*			Not attempted	Not available
787	11 months	6	1:8+++	1:40	27	1:64+++	1:256	Not attempted	Positive
790	8 months	15	1:8 neg.	1:160	*			Not attempted	Not available
898	1½ months	5	1:8 neg.	1:10 neg.	24	1:8 neg.	1:80	Positive	Not available
900	2 years	3	1:8 neg.	1:20 neg.	80	1:4++++	1:80	Positive	Negative
996	1 month	14	1:8 neg.	1:10 neg.	20	1:2 neg.	neg.	Positive	Positive

* Left Hospital before 2nd sample was taken.
At the beginning of this study serums were tested at the initial dilution of 1:8 only.

units of penicillin delayed the death of the embryos for several days.

In summary it can be stated that in all tests the isolated strains behaved like a known strain of ornithosis virus.

Serological tests.—During the winters of 1953–1954 and 1954–1955, serums from 67 children who were suspected of respiratory disease were tested serologically for CF and HI antibodies against ornithosis antigen. The children were from the Hebrew University—Hadassah Medical School Hospital, most of them under one year of age, including three cases from which ornithosis was isolated. Some of the results are summarized in table 4.

It is seen that two of the three cases from which a virus was isolated and who recovered (cases 898 and 900) showed a 4- to 8-fold rise in HI antibodies, with the CF test remaining negative or reaching a low titer (case 900); the third case (996) failed to develop antibodies in both tests. Of the cases examined serologically only, one (case 787) showed a significant rise in titer in both tests. In 4 cases of which a single serum sample only was examined, titers of 160 to 320 were observed in the HI test, accompanied in two cases by a positive CF test.

In order to evaluate the results of the HI test, 100 serums of normal healthy individuals were examined for HI antibodies. It was found that 2% showed a titer of 1:80; 6% a titer of 1:40 and the rest titers of 1:20 and lower. It therefore appears that titers above 1:80 are outside the normal range.

The source of infection for these children could not be traced, except in one case (996) where pigeons which were kept by the family reacted positively with ornithosis antigen in the CF test. In other cases there was no known contact with birds. However, pigeons are found abundantly on the roofs of Jerusalem and must be considered a possible source of infection in all cases.

In order to gain some data on the frequency of ornithosis infection in pigeons, over 100 birds from different lofts in the vicinity of our cases were bled from the wing vein and their blood examined. Twenty-five percent reacted positively with ornithosis antigen in the CF test.

These findings suggest that infection of pigeons with ornithosis virus is common in Jerusalem.

In order to examine the possibility of human to human transmission, serums of family members of patients were tested for the presence of specific antibodies and inquiries were made about the occurrence of respiratory or other diseases in the family. In two cases mothers complained of a respiratory infection prior to the child's illness.

Serums of the parents of 5 positive cases were examined serologically. In

TABLE 5.—*Serological reactions in parents of children with ornithosis infection.*

Case no.	Parents				Serum taken after onset of child's illness	Respiratory disease in family before child's illness
	Mother		Father			
	CF	HI	CF	HI		
38*,†	1:16 +++	neg.	1:8 ++++	neg.	3 months	Not known
62*,†	1:16 +++	—	1:32 ++++	1:80	14 days	None
787	1:8 ++++	1:80	1:8 AC	1:160	46 days	Mother complained of severe cough 10 days before child's illness
996†	1:10 ++++	1:80	neg.	neg.	25 days	Mother had upper respiratory disease before child's birth (child one month old)

* Fatal case.
† Virus isolated.
AC = anticomplementary serum.

four cases one of the parents or both showed specific CF antibodies (table 5).

As the complement fixing antigen is common to various members of the psittacosis-LGV group, it is impossible to relate these single serum titers with certainty to a recent ornithosis infection. It can be stated, however, that infections with LGV virus are very rare in this country and CF antibodies to the group specific antigen are found infrequently; the serums of normal 63 adults, chosen at random, were recently tested with lygranum antigen. Only four serums (6.3%) showed a positive reaction. If these facts are taken into consideration, the positive serological findings in four of five parents of recognized ornithosis cases gain in significance and suggest the occurrence of family infections.

DISCUSSION

The present findings indicate that contrary to prevalent opinion[6] ornithosis infection in very young children and infants is not rare.

It is significant that more cases were diagnosed by virus isolation than by serological methods. Obviously the diagnosis of ornithosis in young children should not be based on serological evidence only, and in suspected cases attempts should be made to isolate the causative agent. The amniotic route of inoculation proved to be a sensitive method for virus isolation.

The difficulties connected with the classification of the various members of this group have been discussed by various authors.[4,5,10,17] The viruses isolated in the present work from patients and an ornithosis virus isolated from sick pigeons in Israel[18] were indistinguishable by all tests used.

A definite rise in the hemagglutination inhibition titer was observed in three cases, of which only one (case 787) showed a concomitant rise in CF antibodies. In four other cases in which only a single sample of serum was available, titers of 160 to 320 were observed in HI tests, accompanied in 2 cases only by positive CF tests. These findings show that the HI test may be of diagnostic value. Further work on the relative sensitivity of the CF and HI tests is indicated.

Antibody titers in the three cases which recovered and from which a

17. Hilleman, M. R., Haig, D. A. and Helmond, R. J. 1951, In vivo and in vitro studies of serological specificity among viruses of the psittacosis-lymphogranuloma venereum group. J. Immunol. **68**: 121–129.
18. Komarow, A. and Goldsmit, L. 1952, The isolation of the causative agent of ornithosis (psittacosis) in Israel. Harefuah, **43**: 83–85.

virus was isolated were low or negative. These findings may be due to the intensive antibiotic treatment[12] given to the children early in the disease, a point which was stressed already by Siegel and others,[9] who noticed a decline in antibody production during the illness after intensive antibiotic treatment.

In 5 out of 6 cases a direct contact with pigeons or other birds could not be demonstrated. In case 996 indirect contact with piegons showing CF antibodies for ornithosis virus was definitely possible, as the child had stayed for some time with relatives who kept pigeons found serologically positive.

Serological studies carried out on blood from over 100 pigeons from different lofts in Jerusalem showed that 25% of the serums reacted with the CF antigen prepared from ornithosis virus. The possibility must therefore be considered that pigeons constitute a source of infection for humans.

The question of human to human transmission in psittacosis has recently been discussed by Meyer,[5] who states that although the evidence is scarce, it seems likely that human psittacosis may be spread through subclinically infected humans. The presence of antibodies in the serums of the parents and a respiratory infection in two mothers prior to their children's illness supports this suggestion.

SUMMARY

1. Virus strains belonging to the psittacosis-lymphogranuloma group were isolated from 6 cases of atypical pneumonia in children. Three strains were isolated from whole blood and three from autopsy material. Among the criteria used for the classification of these strains as ornithosis virus was their pathogenicity for mice and pigeons by the intracerebral route and their immunological relationship with ornithosis virus shown in cross protection test.

2. Inoculation of chick embryo by the amniotic route was found to be a sensitive method for virus isolation.

3. Serological tests for ornithosis virus were carried out on a number of children with atypical pneumonia. In one case a concomitant rise in complement fixing and hemagglutination inhibiting antibodies was observed. In another case (one-month-old child), from whom a virus was isolated, there was a fourfold rise in hemagglutination inhibiting antibodies only, while the complement fixation test remained negative. In 4 cases where only a single sample was available, titers of 160 to 320 in hemagglutination inhibiting antibodies were obtained, a range of titers not observed in normal serums. In two of these cases the complement fixation test was also positive.

4. Antibodies specific for the psittacosis-lymphogranuloma group were found in the serums of four parents of these cases.

5. Over 100 pigeons from lofts in the vicinity of these cases were tested serologically: 25% were found positive in the complement fixation test.

The epidemiological significance of these findings is discussed.

SEROLOGICAL EVIDENCE OF TOXOPLASMOSIS IN ANIMALS

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Toxoplasmosis as a human disease was first demonstrated in 1939 by Wolf and his co-workers in New York.[1] These workers recovered an infectious agent from a fatal case of encephalomyelitis which was shown by animal inoculation and by serological tests to be a strain of *Toxoplasma gondi*, a parasite first encountered in African rodents by Nicolle in 1908.[2] The number of proved human cases reported to date are relatively few, numbering less than 50. However, on the basis of information obtained in serological surveys it seems probable that subclinical toxoplasmosis is a frequent occurrence affecting as many as 20 to 40% of the adult population in this country.[3–4]

Nothing concrete is known of the source of human infections. The parasite occurs in many mammals and in pigeons, as well as in arthropods.[5] If animals are important in the epidemiology of human toxoplasmosis, it is well to have information concerning the incidence of infection in these hosts. This study was undertaken to obtain such information.

MATERIALS AND METHODS

Practically all the serums used in the current study were obtained from vertebrates found in the Middle Atlantic States. Serums were drawn from dogs on the day of delivery to the Army Medical Service Graduate School, from farm areas in southern Pennsylvania, from pigeons trapped in Washington, D. C., and from a variety of wild animals caught in the Patuxent Refuge in Laurel, Maryland. In addition, sera collected from rice rats trapped in the vicinity of Charleston, South Carolina, were also tested.

Complement-fixation tests employing overnight incubation at 4 C with 2 kinds of antigens prepared from toxoplasma-infected chick embryos were performed to demonstrate antibody. The antigen used in the early tests was prepared from toxoplasma-infected chorioallantoic membranes of chick embryos by the method of Warren and Russ.[6] This antigen is tedious to produce since the yield is generally small. Later it was found that an equally satisfactory antigen could be prepared readily from the allantoic fluid of 9-day embryonated eggs infected by the allantoic route with the RH strain of toxoplasma. Six days after inoculation each egg yielded 5 to 10 ml of fluid which could be used at a 1:8 dilution to provide 2 units of antigen. The potency of the antigen was preserved by lyophilization. The new antigen was employed in later tests.

In table 1 are shown results of tests comparing the activity of the new allantoic fluid antigen with the standard chorioallantoic membrane extracted preparation. It is seen that the potency of the two antigens is similar. Further, essentially identical serum titers were obtained when toxoplasma immune guinea pig serum was tested with

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1. Wolf, A., Cowen, D. and Paige, B. H. 1939, Human toxoplasmosis; occurrence in infants as an encephalomyelitis. Verification by transmission to animals. Science, **89**: 226–227.
2. Nicolle, C. and Manceaux, L. 1908, Sur une infection a corps de Leichman (ou organismes voisins) du gondi. Compt. rend. Acad. d. sci. **147**: 763–766.
3. Feldman, H. A. 1953, The clinical manifestations and laboratory diagnosis of toxoplasmosis. Am. J. Trop. Med. & Hyg. **2**: 420–428.
4. Weinman, D. 1952, *Toxoplasma* and toxoplasmosis. Ann. Rev. Microbiol. **6**: 281–298.
5. Giroud, P., LeGac, P. and Gaillard, J. A. 1952, Mise en evidence de Toxoplasmes sur souris inoculees avec des broyats de *Trombicula irgaci* (Marc Andre 1950) recueillis sur *Lemniscomys barbarus striatus* et sur *Mylomys cuninghamei alberti* captures en Oubangui-Chari. Bul Soc. path. exot. **45**: 449–451.
6. Warren, J. and Russ, S. B. 1948, Cultivation of *Toxoplasma* in embryonated eggs. An antigen derived from chorio-allantoic membrane. Proc. Soc. Exper. Biol. & Med. **67**: 85–89.

TABLE 1.—*Results of grid titrations to compare allantoic fluid toxoplasma antigen with chorioallantoic membrane extract material.*

Guinea pig serum	Serum dilution	Antigen dilution							
		Allantoic fluid				Chorioallantoic membrane			
		1:8	1:16	1:32	1:64	1:8	1:16	1:32	1:64
Toxoplasma immune	1:80	4*	4	3	0	4	4	3	0
	1:160	4	4	1	0	4	4	1	0
	1:320	4	2	0	0	4	4	0	0
	1:640	4	0	0	0	4	2	0	0
	1:1280	0	0	0	0	2	1	0	0
Normal	1:40	0	0	0	0	0	0	0	0

* Numbers indicate degree of fixation of complement in the test.
4 = complete fixation, 1, 2 or 3, partial fixation; and 0, lack of fixation.

the 2 preparations using 2 units of antigen in each instance. Fixation was not obtained with either antigen when tested against a series of dog and human serums known to contain complement-fixing antibody against distemper, mumps, herpes and psittacosis viruses and epidemic typhus rickettsia, as well as convalescent serums from 10 patients with clinically diagnosed cat scratch fever.

RESULTS

Complement-fixation tests for *Toxoplasma* were performed with serums of 426 animals belonging to 11 different species. The findings summarized in table 2 show that 25% of 180 dogs and 19% of 107 wild rabbits had detectable antibody, but such antibody was not found in the serums of the remaining animal species. It will be noted further that antibody titers in the positive serums were appreciable, ranging from 1:8 to 1:128.

A serum titer of the order of 1:128 suggested to us that infection with *Toxoplasma* in the serologically positive animal might have been of recent origin. In this connection attempts were made to isolate *Toxoplasma* from brain and visceral tissues of 2 dogs with serum titers of 1:64 and 1:128. The results of both attempts were negative.

DISCUSSION

It is possible that the complement-fixing substances in serums reacting with toxoplasma antigen are due to infections with agents other than *Toxoplasma*, but there is no evidence at hand suggesting that this is so. The only organism demonstrated to be antigenically related to *Toxoplasma* is *Sarcocystis*.[7] The positive serums in the current study have not been tested against sarcocystis antigen since this material was not available. However, it has been demonstrated by others[8] that

7. Muhlpfordt, H. 1951, Das Verhalten Sarcosporidien-infizierter Tiere im Sero-farbtest auf Toxoplasmose nach Sabin-Feldman. Ztschr. Tropenmed. Parasitol. **3**: 205–215.
8. Awad, F. I. 1954, A new dye test for *Toxoplasma* and *Sarcocystis* infections by use of *Sarcocystis tenella* spores. Tr. Roy. Soc. Trop. Med. & Hyg. **48**: 337–341.

TABLE 2.—*Toxoplasma complement-fixing antibody in domestic and wild animals.*

Species	Number tested	Number positive	CF titer				
			1:8	1:16	1:32	1:64	1:128
Dog	180	45	6	18	13	6	2
Wild rabbit	107	20	8	8	3	1	0
Cat, domestic	8	0					
Fox	3	0					
Marmot	12	0					
Opossum	12	0					
Pigeon	14	0					
Racoon	14	0					
Rat, Norway	64	0					
Rat, Rice	5	0					
Squirrel	7	0					

the toxoplasma complement-fixing reaction, unlike the dye test of Sabin and Feldman,[9] does not give positive results with sarcocystis serums. It may be mentioned here that complement-fixing antibody against *Toxoplasma* was present at a titer of 1:8 in the serum of 1 of 9 men who regularly handle dogs at the Army Medical Service Graduate School, but such antibody was not present in any of 38 serums obtained from normal adults in the Washington area. The absence of toxoplasma complement-fixing antibody in the serums of the 38 normal adults is not unusual in light of the observation of Feldman of wide variation in the incidence of toxoplasma dye test antibody in the adult population in different geographic regions.[3] Jacobs studied serums from 28 suspected cat scratch fever patients and found 2 with significantly high toxoplasma dye test antibody titers: from the enlarged axillary lymph gland of one of these patients toxoplasmas were recovered.[10] Among 10 patients in our series with clinically diagnosed cat scratch fever none had detectable complement-fixing antibody against *Toxoplasma*. These serums were not examined by the dye test technique. Nevertheless, it appears that toxoplasmas were not etiologically concerned with the illnesses in our cases.

If the complement-fixing antibody demonstrated in the animal serums indicates experience with *Toxoplasma*, then infection is extremely common in dogs and only slightly less so in wild rabbits. Absence of antibody in the serums of other animals included in the tests does not, of course, mean that infection may not be of frequent occurrence in these species also. Indeed, spontaneous toxoplasma infection has been demonstrated in at least one of these species by Jacobs who isolated the parasite from pigeons trapped in the Washington area.[11]

The question as to the mechanism of spread of infection from animal to man or vice versa is still unanswered. Weinman has shown recently that toxoplasmosis may be transmitted to pigs and rats by feeding upon infected tissues.[12] These results suggested to Weinman that man may acquire infection from inadequately cooked pork in much the same manner as trichinosis is contracted. Such an explanation might account for the reactions for the positive results in our dogs but seems unlikely when applied to the rabbits. Some other means, in addition to oral infection, probably is active in nature by which toxoplasmas are disseminated.

SUMMARY

It has been demonstrated that allantoic fluid of toxoplasma infected chick embryos contains substances which fix complement specifically with toxoplasma immune serum. Further, it appears on the basis of serological data that toxoplasma infection occurs in an appreciable number of dogs and wild rabbits found in portions of the middle Atlantic states.

9. Sabin, A. B. and Feldman, H. A. 1948, Dyes as microchemical indicators of a new immunity phenomenon affecting a protozoan parasite (*Toxoplasma*). Science, **108**: 660–663.
10. Armstrong, C. and MacMurray, F. G. 1953, Toxoplasmosis found by recovery of *Toxoplasma gondii* from excised axillary gland. J.A.M.A. **151**: 1103–1104.
11. Jacobs, L., Melton, M. L. and Jones, F. E. 1952, The prevalence of toxoplasmosis in wild pigeons. J. Parasitol. **38**: 457–461.
12. Weinman, D. and Chandler, A. H. 1954, Toxoplasmosis in swine and rodents. Reciprocal oral infection and potential human hazard. Proc. Soc. Exper. Biol. & Med. **87**: 211–216.

THE COMPARATIVE PATHOLOGY OF VENEZUELAN EQUINE ENCEPHALOMYELITIS

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These investigations are concerned with the pathological effects of the Venezuelan equine encephalomyelitis (VEE) virus in mice, guinea pigs, rabbits and monkeys (*Macacus rhesus*). The only other description of pathological changes in VEE is that of Gilyard,[1] who studied the disease in the mule. The unusual finding revealed by the present studies on the comparative pathology is that a virus may produce generalized necrosis of myeloid and lymphoid tissues, as severe as that observed following intense radiation, and that the same virus may be both lymphomyelotropic and neurotropic in different hosts.

METHODS

Animals infected by injection of or by exposure to an aerosol containing VEE virus which had been propagated in chick embryo were examined after dying or after being sacrificed when they survived exposure. Virus in the blood of human patients or of guinea pigs was also injected into guinea pigs or mice. Tissues examined included heart, great vessels, such as aorta and pulmonary vein, lungs, spleen; lymph nodes or lymphatic tissue from the eyelid, neck, trachea, bronchi, axilla, groin, and mesentery, as well as Peyer's patches; adrenal, kidney, parathyroid, pituitary, bone marrow, eyes, and retro-orbital tissue; and central nervous system, including cerebral cortex, thalamus, pons, medulla and thoracic spinal cord. Except for the nervous system and bones, all tissues were fixed in Zenker's fluid. Neural tissue was fixed in 10% formalin, bone of mouse in Bouin's, and bone of other species in 10% formalin. Decalcification of bones of larger animals was performed with 5% nitric acid. Bouin's-fixed and the decalcified tissues were stained with hematoxylin and eosin. The others were stained with a modified Lillie-Giemsa method.

RESULTS

Dosage, route of administration, immunization procedures, survival in days and incidence of significant lesions for guinea pig, rabbit, mouse and monkey are recorded in table 1.

Lesions in guinea pigs after infection with chick embryo grown virus

After death from infection with VEE virus, all lymph tissues and nodes were congested, red, soft, and only slightly enlarged. Necrosis was widespread in lymphoid and myeloid tissue of all lymph nodes, spleen, thymus, intestinal and conjuctival lymphoid tissue, and bone marrow (pl. 1a, 1b, 2a, 2b, 3b, and 4). The spleen was slightly enlarged, deeply cyanotic and turgid. Microscopically, sinusoids were empty and the pulp cords were heavily engorged by red blood cells. Almost no white blood cells were present (pl. 3a). The Malpighian follicles showed central necrosis (pl. 3b). The spleen resembled the active hyperemia seen in congenital hemolytic anemia of humans, except for the necrosis in the Malpighian follicles. Encephalomyelitis was never found

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1. Gilyard, R. T. 1945, Clinical study of Venezuelan virus of equine encephalomyelitis in Trinidad, B.W.I. J. Am. Vet. Med. Assoc. **106**: 267–277.

TABLE 1.—*Lesions of VEE infection in guinea pigs, mice, rabbits and monkeys.*

Host	Route of infection	Number of animals	Immunizing dose	Dose mouse IC LD_{50}	Days		Ratio showing lesions			
					Survival	Sacrificed	Lymph node necrosis	Spleen necrosis	Bone marrow necrosis	Encephalomyelitis
Guinea pig	Respiratory	18		30–57,550	3–6, 12		18/18	18/18	18/18	0/18
		2	1.0	8900–57,550		15	0/2	0/2	0/2	
		1	0.1	8,900	8		0/1	0/1	0/1	0/1
		3	0.01	8900–57,550	5–6	15	0/3	0/3	0/3	1/1
		2	0.001	8900–57,550	6		2/2	2/2	2/2	
	Subcutaneous	7		50,000	3–4		7/7	7/7	7/7	
		6	1.0	50,000		3–4	0/6	0/6	0/6	
	Intraperitoneal	2		1– 10	3–4		2/2	2/2	2/2	0/2
Rabbit	Respiratory	10		30–19,800	2–4		10/10	10/10	10/10	0/10
Mouse	Respiratory	5			6		3/5	3/5	0/3	2/5*
	Intraperitoneal	6		1– 100	4–5		6/6	6/6	4/6	0/6
Monkey	Respiratory	1		1,050		22	0/1	0/1	0/1	1/1
	Subcutaneous	1		1,000		51	0/1	0/1	0/1	1/1
	Intraperitoneal	2		1,000		9–22	0/1	0/1	0/1	2/2
	Intracerebral	1		10,000		8	0/1	0/1	0/1	1/1

* Three of 5 mice showed lymph node and splenic necrosis. The other 2 had only encephalomyelitis.

except in 1 of 78 immunized and subsequently infected guinea pigs, the remainder surviving without lesions. This guinea pig had been immunized by intradermal injections of 0.1 ml of 1:10 dilution of VEE vaccine[2] at 3 weekly intervals. Death with encephalomyelitis occurred 8 days after infection by aerosol. Atrophy of the lymph nodes, spleen and bone marrow were the only other lesions.

Blood count changes in guinea pigs after infection with chick embryo grown virus

Because of the extensive necrosis in the hematopoietic system, hematological studies were made in guinea pigs infected with VEE virus. The virus was injected subcutaneously in a dose of 10^4 mouse IC LD_{50}'s into vaccinated and normal guinea pigs. Before and at daily intervals after injection, red blood cell, white blood cell and differential leucocyte counts were determined. Figure 1 records the results of the changes in white blood cell counts. Initially the white blood cell counts ranged from 4900 to 14,400/cmm. On the second day after infection with VEE virus, all infected guinea pigs showed a sharp decrease in white blood cells, the total count ranging from 800 to 2800/cmm, the low counts persisting until death. The absolute number of lymphocytes decreased significantly on the 1st day, while the number of polymorphonuclear leucocytes increased slightly. On the 2nd day, both polymorphonuclear neutrophils and lymphocytes decreased. The infected animals died in 3 to 4 days. The immunized guinea pigs did not die and showed either no change in white blood cell count or an increase for only the first 2 days. No changes in red blood

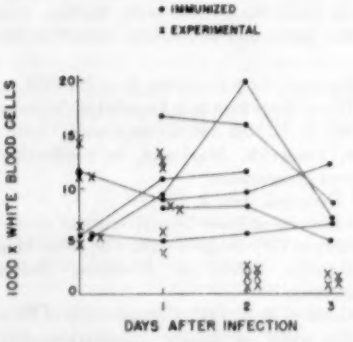

FIG. 1.—White blood cell counts after infection of guinea pigs with Venezuelan equine encephalomyelitis virus.

2. Randall, R., Mills, J. W. and Engel, L. L. 1947, The preparation and properties of a purified equine encephalomyelitis vaccine. J. Immunol. **55**: 41–52.

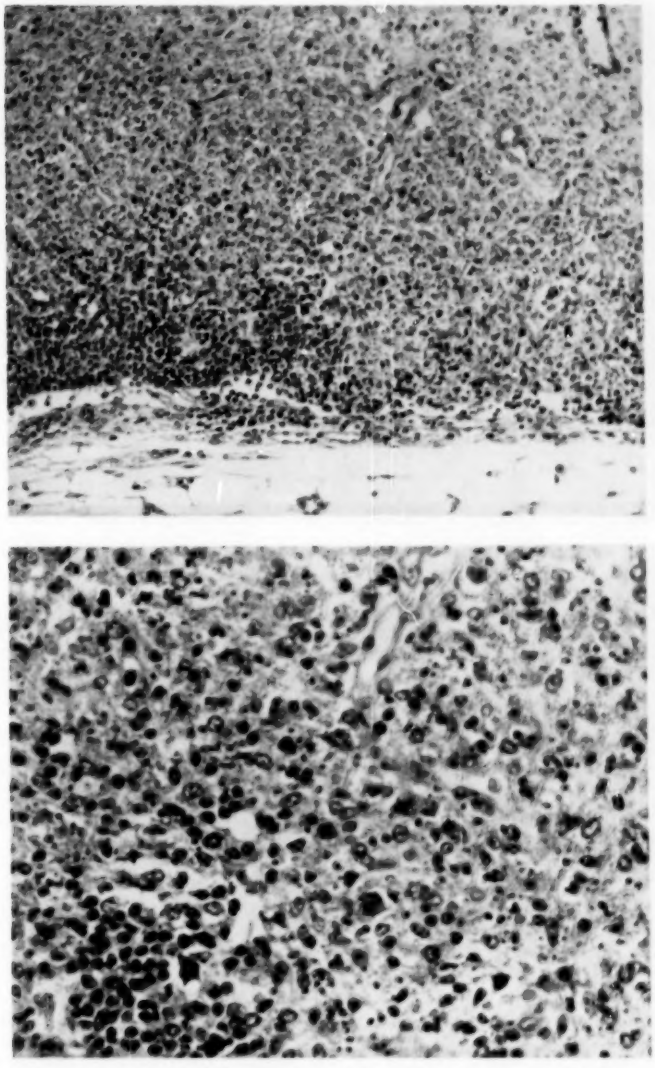

PLATE 1a (top).—Guinea pig 51-147. Lymph node. Necrosis of lymphocytes in the cortex. Capsule stroma, and reticulum are intact. Giemsa stain. 190×.

1b (bottom).—Guinea pig 51-147. Necrosis in the field illustrated above. 420×.

cell counts occurred in either group. These hematological observations confirm the pathological findings that destruction of lymphoid and myeloid elements of the guinea pig by VEE infection decreased the circulating white blood cells within 2 days after infection. The extraordinary decrease in white blood cells appears to be characteristic of VEE infection, and would be a useful diagnostic and experimental test for obtaining evidence of the infection in 2 days, instead of waiting 3 to 7 days for death of the host.

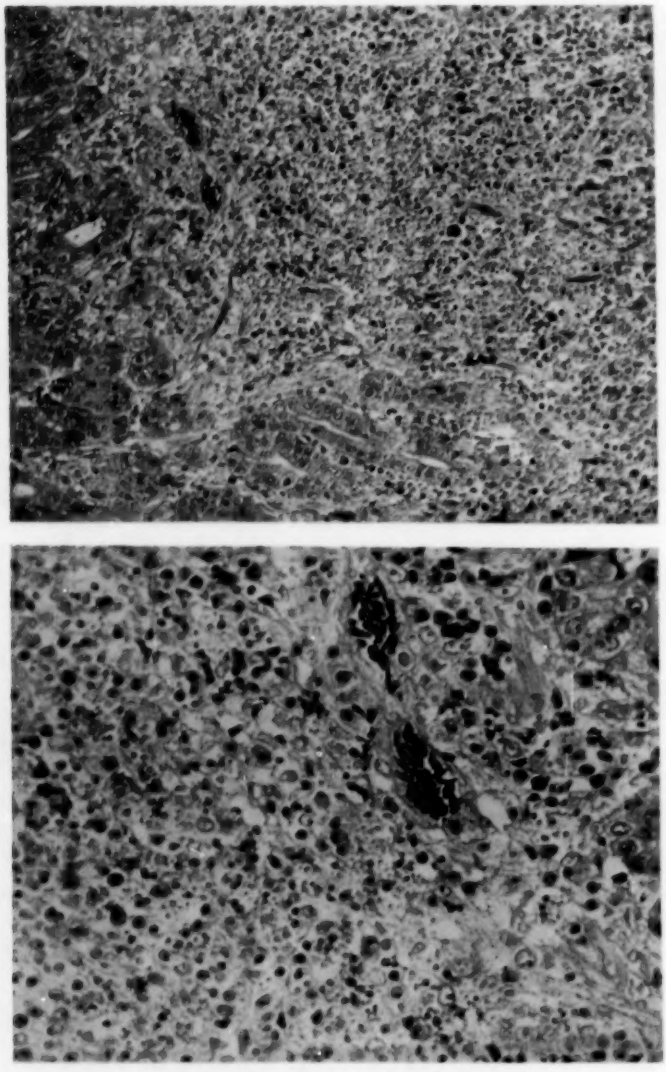

PLATE 2a (top).—Guinea pig 51-147. Peyer's patch. Necrosis of lymphocytes. Capillary congestion and intact mucosa and stromal cells are evident. Giemsa stain. 190×.

2b (bottom).—Guinea pig 51-147. Mucosal and lymphoid junction of field illustrated above. 420×.

Lesions in the mouse infected with chick embryo grown virus

Mice dying 4 to 5 days after infection with virus showed lesions involving lymphoid and myeloid tissues essentially like those in the guinea pig and rabbit (table 1). At 6 days, 2 of 5 showed encephalomyelitis and atrophy of the lymphoid tissues as evidenced by shrunken lymph nodes having reduced number of lymphocytes and follicles.

Encephalomyelitis was characterized by perivascular infiltration of lymphoid and polymorphonuclear leucocytes in

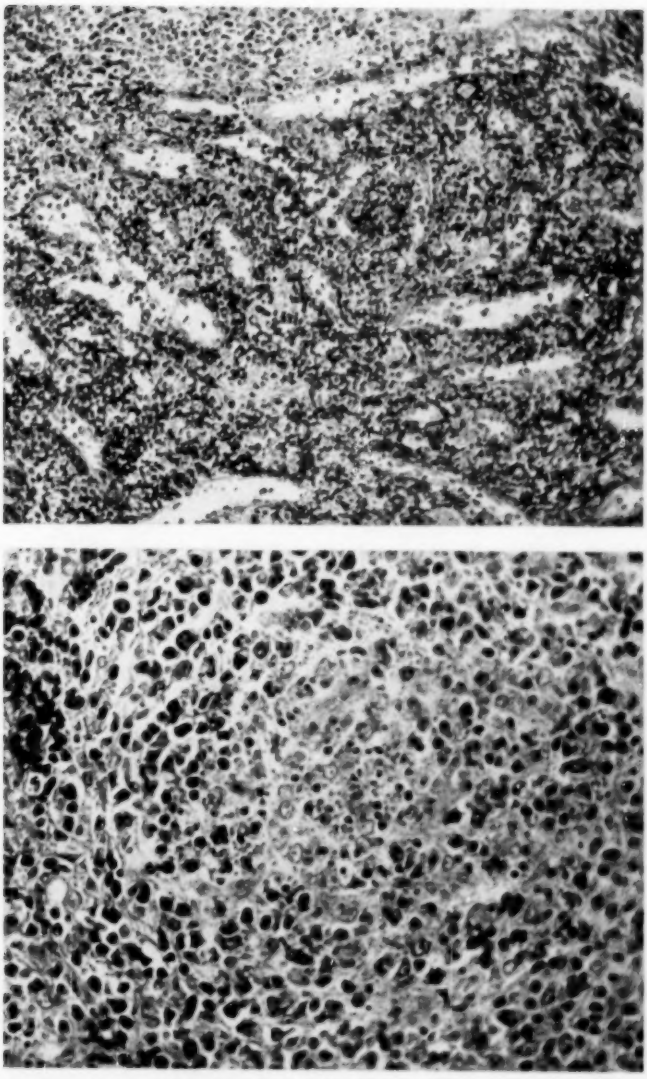

PLATE 3a (top).—Guinea pig 51-147. Spleen. Hemorrhage or engorgement of pulp and empty sinusoids. Almost no leucocytes in the pulp. Giemsa stain. 190×.

3b (bottom).—Guinea pig 51-147. Spleen. Necrosis in the germinal center of a Malpighian follicle. 475×.

all parts of the central nervous sytem (pl. 5, 6, and 7). However, most of the lesions occurred in the cerebral cortex, in both grey and white matter, and in the pons. Other lesions were necrosis of nerve cells with shrinkage and phagocytosis of the cells by polymorphonuclear and mononuclear leucocytes, plaques of degenerating pale staining nerve cells with decreased Nissl substance in both cortex and spinal cord, and plaques of infiltrating necrotic

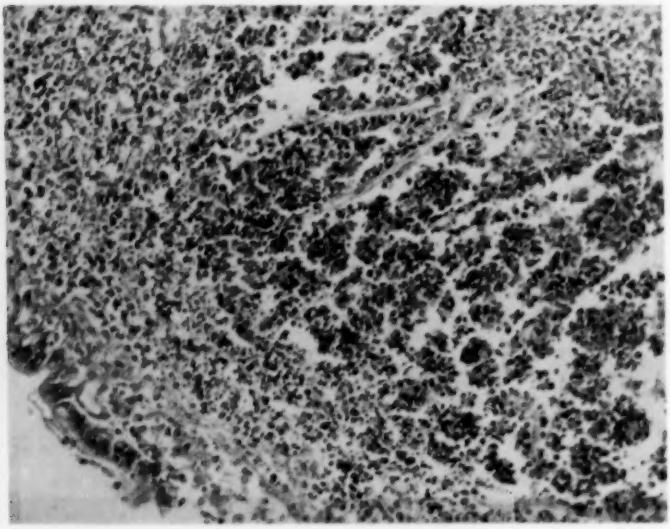

Plate 4.—Guinea pig 51-307. Necrosis in subconjunctival lymphatic nodule. Giemsa stain. 190×.

PLATE 5.—Mouse 52-297. Brain. Cerebral cortex. Engorgement of capillaries and small blood vessels, infiltration by round cells and neutrophils, and shrinkage of ganglion cells with edema spaces about them. Giemsa stain. 190×.

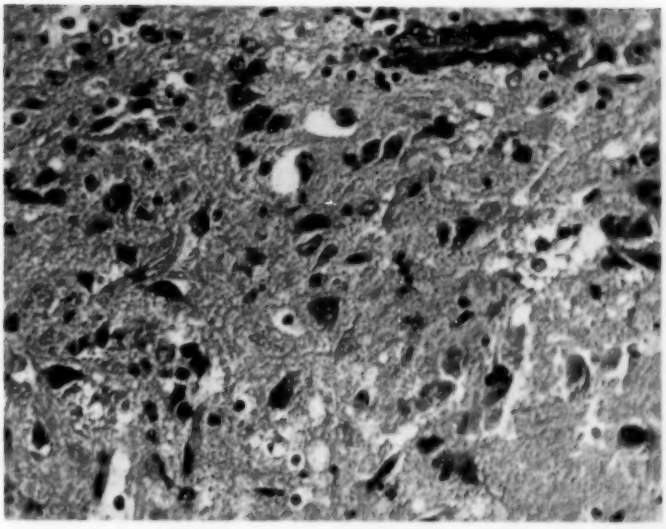

PLATE 6.—Mouse 52-297. Spinal cord. Clusters of degenerating ganglion cells showing karyolysis and diminished Nissl substance. Leucocytic infiltration about the central canal and ganglion cells. Hematoxylin and eosin stain. 420×.

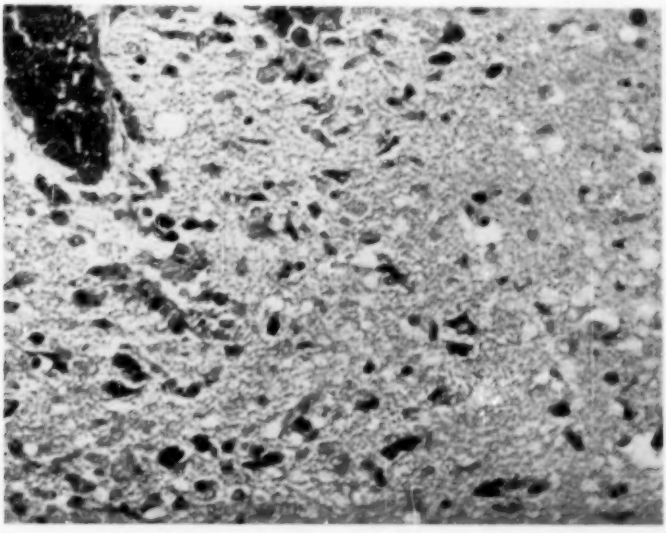

PLATE 7.—Mouse 52-297. Brain. Infiltration of cortical tissue. Pyknosis and fragmentation. Giemsa stain. 420×.

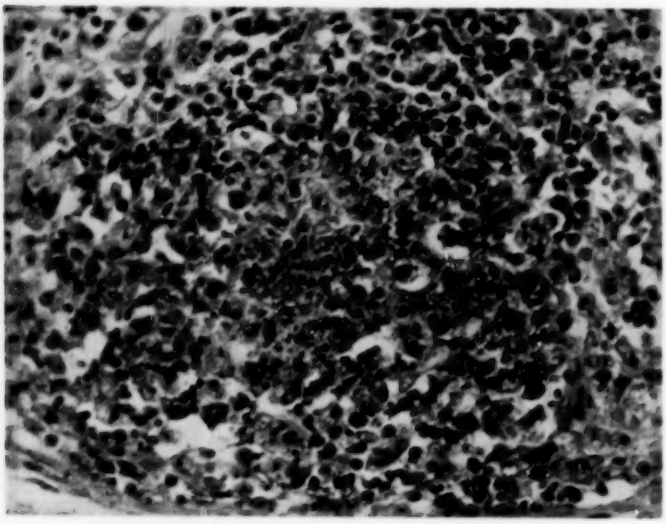

PLATE 8.—Rabbit 51-219. Lymph node. Necrosis in germinal centers and pulp. Reticulum and stroma are intact. Giemsa stain. 420×.

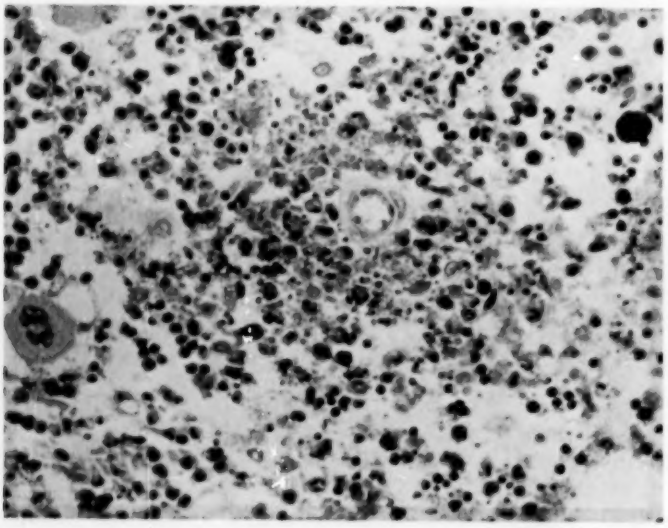

PLATE 9.—Rabbit 51-218. Bone marrow necrosis. Giemsa stain. 420×.

leucocytes in both grey and white matter of brain and cord.

Lesions in rabbit infected with chick embryo grown virus

Rabbits died 2 to 4 days after infection with VEE virus. The lesions were similar to those of the guinea pig, with extensive necrosis of lymphoid and myeloid tissues (pl. 8 and 9). The splenic changes were also the same as those observed in the guinea pig.

Lesions in monkey infected with chick embryo grown virus

Five monkeys infected in different ways, indicated in table 1, became ill for about 6 to 8 days. None died but they were sacrificed 8 to 51 days after exposure. All showed encephalitis with the most extensive lesions in the oldest case. None had changes in the spinal cord. Swelling of endothelium lining small blood vessels, mainly capillaries, and perivascular infiltration by mononuclear cells with scant cytoplasm were the dominant changes (pl. 10). In some instances the infiltrated areas were circular nodules of mononuclear cells resembling a granuloma. Atrophy and degeneration with multiplication of sarcolemma nuclei was found in the sartorius muscle of one monkey, 9 days after intraperitoneal injection of 1000 guinea pig IP LD_{50}'s.

Infection produced by blood of mammals infected with VEE virus

The incidence of encephalomyelitis in mice and guinea pigs infected with egg-propagated VEE virus was very low in the experiments cited above. However, the blood of 5 humans ill after injection with VEE vaccine[3] uniformly produced encephalomyelitis 5 to 6 days after intraperitoneal injection in mice.

3. Sutton, L. S. and Brooke, C. C. 1954, Venezuelan equine encephalomyelitis due to vaccination in man. J.A.M.A. **155**: 1473–1476.

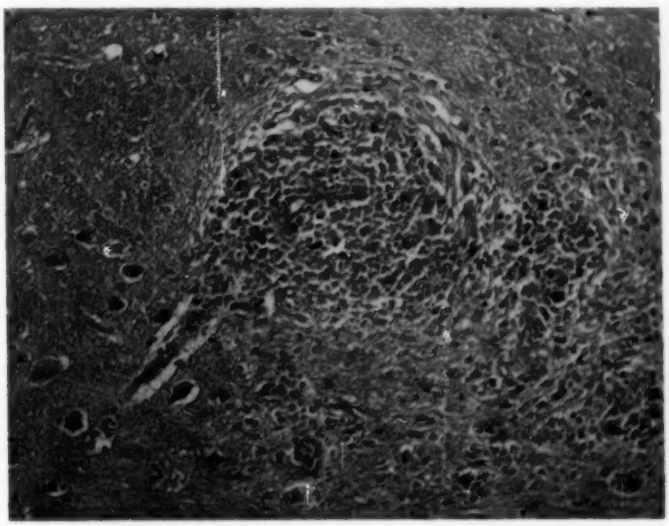

PLATE 10.—Monkey 52-1356. Brain. Perivascular masses of mononuclear and lymphoid cells, endothelial swelling, degenerating ganglion cells and edema 51 days after aerosol exposure. Giemsa stain. 190×.

In order to test the effect of virus grown in mammals, further observations were obtained from mice or guinea pigs injected intraperitoneally with infected guinea pig blood. It seemed possible that consistent production of encephalomyelitis in mice might depend upon the mode of propagation of the virus. The following experiments demonstrate that the occurrence of encephalomyelitis depended not only on the recipient host and the method of propagation of the virus, but also on the survival interval after infection.

Encephalomyelitis produced in mice by injection of guinea pig blood infected with VEE virus

Guinea pigs were inoculated intraperitoneally with chick embryo grown VEE virus. Blood was collected at 15 and 51 hours after inoculation to obtain trace and high titered virus samples, respectively. These two blood samples were passed in parallel intraperitoneally into guinea pigs and titrated in mice. This procedure was repeated for 6 serial passages from guinea pig to guinea pig, with titration in mice at each passage. Pathological studies were made of the guinea pigs and mice from the 15-hour passage series. The 51-hour series produced overwhelming infections. All the guinea pigs in the 15-hour series showed the same lymphoid and myeloid changes as previously described. There was no encephalomyelitis. On the other hand, the titration mice showed encephalomyelitis and lymphoid and splenic changes as indicated in table 2. Encephalomyelitis appeared more regularly 6 days or more after infection, whereas lymphoid and splenic changes, either necrosis or atrophy, were not as frequent as encephalomyelitis. Furthermore, with passage through guinea pig blood there was a higher incidence of encephalomyelitis than was noted with chick embryo grown virus.

TABLE 2.—*Mouse encephalomyelitis and lymphoid and splenic necrosis or atrophy after intraperitoneal inoculation with blood of guinea pigs infected with VEE.*

Transfer to		Days after transfer	Encephalomyelitis incidence	Lymph node and spleen	
Guinea pig	Mouse			Necrosis	Atrophy
1	1	4	3/6	5/6	1/6
		5	5/5	4/4	0/4
		6	10/10	8/10	2/10
		7	3/3	2/3	1/3
2	1	5	1/2	0/2	2/2
		6	4/4	0/4	4/4
		7	7/8	0/8	7/8
3	1	4	1/1	0/1	1/1
		5	1/1	0/1	0/1
		7	6/7	1/7	4/7
		8	3/3	0/3	3/3
4	1	4	1/1	0/1	0/1
		5	5/5	2/5	0/5
		6	4/4	2/4	0/4
5	1	5	0/1	1/1	0/1
		6	4/4	2/4	1/4
6	1	5	1/1	0/1	0/1
		6	4/4	3/4	1/4
Total			63/70	30/69	27/69

Lesions in guinea pigs after injection with guinea pig blood infected with VEE virus

All of 8 guinea pigs inoculated intraperitoneally with chick embryo grown VEE showed the characteristic lymphoid and myeloid necrosis, engorgement in the splenic pulp and empty splenic sinusoids noted above. In the 6 consecutive guinea pig passages with guinea pig blood from the 15-hour series, 34 animals dying in 2 to 5 days after intraperitoneal injection showed no significant differences in lesions from those observed in the guinea pigs infected with embryo grown virus. However, 11 of the 34 guinea pigs showed adrenal hemorrhages, most severe in the zona intermedia, and occasionally necrosis of adrenal cortical cells in the hemorrhagic areas. In only 9 guinea pigs was lymphoid tissue found in the eyelid and this lymphoid tissue invariably showed necrosis similar to that occurring in lymph nodes.

DISCUSSION

A comparative study of the pathological effects of VEE virus shows unequivocal viscerotropic effects of this agent on the lymphomyelopoietic sys-

TABLE 3.—*Effects of aerosol infection with VEE virus in mouse, guinea pig, rabbit and monkey.*

	Respiratory LD_{50} in mouse IC LD_{50}	Survival days	Sacrificed days	Leucopenia day	Encephalomyelitis	Necrosis		
						LN	Spleen	BM
Rabbit	14	2–5		?	0	+	+ Active hyperemia	+
Guinea pig	30	3–6		2 after IP injection	0	+	+ Active hyperemia	+
Mouse	1.3×10^3	5–9		?	+	±	±	±
Monkey	$1.05 \times 10^{3*}$		8–51	0	+	0	0	0

* The smallest aerosol dose was 1.05×10^3 mouse IC LD_{50}; the respiratory ID_{50} has not been determined but must be less than this value.

tems of the guinea pig and rabbit and neurotropic effects in the mouse and monkey independent of the route of inoculation. So far as we are aware, such a sharp distinction of effects of a virus has not been seen or recognized, although we are fully aware of the viscerotropic and neurotropic properties of the yellow fever virus. The effects of the yellow fever virus depend on the site of injection of the virus and not upon the natural propagation of it in the host.[4]

Species differences are found also in sensitivity to infection by various routes. In special studies by one of us (D.G.S.), the respiratory LD_{50} for the rabbit was found to be 14 mouse IC LD_{50}'s for the guinea pig 30, and for the mouse 1370. The monkey has also been infected by the aerosol route with an inhaled dose equivalent to 1050 mouse IC LD_{50}'s. Since this was the smallest respiratory dose tested in monkeys, the ID_{50} for this species may be much lower. Thus, the monkey appears to be more sensitive than the mouse. In spite of an apparent greater susceptibility of the monkey to aerosol infection, the monkey survives infection by any route, whereas the mouse invariably succumbs. The infectious dose, therefore, appears to be unrelated to the size of the animal host, but directly related to the host species. The lethal dose also depends upon the host species and not on the infectious dose.

The different types of lesions found in the different species (table 3) may be related to the duration of survival after infection. There is a possibility that guinea pigs and rabbits showing extensive necrosis of the lymphomyelopoietic system did not live long enough to develop lesions in the central nervous system. Only 1 of 78 guinea pigs, infected subsequent to vaccination, developed encephalitis and this animal also had the longest survival period. The incidence of encephalomyelitis was greater in the mice with the longest survival after infection. Furthermore, mice dying early, 4 to 5 days, had more necrosis of the lymphomyelopoietic system than those living longer. In the monkey, although ill with fever, anorexia, tremors and convulsions lasting up to 6 days after infection, leucopenia was not observed. Whether the monkey also undergoes a cycle of necrosis of the lymphomyelopoietic tissue in the early stages of infection remains to be determined. The occurrence of central nervous system lesions seems, therefore, to be related to the survival period after infections.

SUMMARY AND CONCLUSIONS

Venezuelan equine encephalitis virus infects the mouse, guinea pig, rabbit and monkey. The first three species are

4. Theiler, M. 1930, Studies on the action of yellow fever virus in mice. Ann. Trop. Med. & Parasitol. **24**: 249–272.

invariably killed by the infection, but monkeys survive. The survival periods for the rabbit, guinea pig, mouse and monkey increase in the order cited. Infected monkeys have been studied 8 to 51 days after exposure, and all had to be sacrificed for autopsies.

Pathological changes are found in the lymphomyelopoietic and central nervous systems. The guinea pig and rabbit show changes only in the former, the mouse in both, and the monkey only in the latter. The lymphomyelopoietic lesions consist of necrosis of all lymphoid and myeloid tissues, with a unique lesion in the spleen characterized by empty sinusoids, severe congestion in the pulp cords and necrosis of Malpighian follicles. Encephalomyelitis consists of perivascular infiltrations in all parts of the brain and spinal cord, but most frequently in the grey matter. Necrosis, shrinking and phagocytosis of nerve cells occur occasionally. Encephalitis is found 8 to 51 days after infection in monkeys. In mice dying of the infection, encephalomyelitis increases in frequency and severity after 5 days, when the necrosis of the lymphomyelopoietic tissues decreases. The largest brain lesions occur in the monkey, yet these are too small to be detected grossly.

The blood of the guinea pig shows extreme leucopenia 2 days after subcutaneous injection of the virus.

Comparative pathologic studies of infection with this virus reveal the unusual observation that lymphomyelopoietic necrosis is caused in the guinea pig, rabbit, and mouse, while encephalomyelitis is produced in the mouse and monkey. The infectious and the lethal doses appear to be unrelated to the size of the animal host, but directly related to the host species.

EXPERIMENTAL STUDIES ON ARGASID TICKS AS POSSIBLE VECTORS OF TULAREMIA

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Since Parker, Spencer and Francis (1924) reported the transmission of *Pasteurella tularensis* by naturally infected *Dermacentor andersoni*, numerous articles on infection in or transmission by different species of ixodid ticks have been published. A detailed review of the literature was presented by Steinhaus (1946) and more recent studies are reviewed in the publication of Hopla (1953).

Concerning infection of argasid ticks with *Past. tularensis*, however, few articles appear in the literature despite the fact that numerous mammals and birds which are known to be naturally infected with *Past. tularensis* (Burroughs et al, 1945) serve as hosts of argasid ticks, especially of the genus *Ornithodoros*. Kamil and Bilal (1938) reported transmission of *Past. tularensis* by experimentally infected *Ornithodoros lahorensis*. Davis (1940), in studies of ticks of the genus *Ornithodoros* as potential vectors of various infectious agents, found that *Past. tularensis* may persist in the tissues of *O. turicata* and *O. parkeri* for at least 674 days and 701 days, respectively. In no instance, however, did the ticks transmit the organisms during their process of feeding. The same author (Davis, 1945) stated that *Past. tularensis* may be transmitted experimentally by *O. moubata*. The infection proved to be fatal for ticks of this species as well as for *O. nicollei*, in which the organisms persisted for 207 days (Davis, 1943).

Recently Burgdorfer and Pickens (1954) reported preliminary results on the infection of *O. moubata* and *O. turicata* with *Past. tularensis*. The ticks were infected by feeding on the air-sac membrane of inoculated embryonated chicken eggs. The organisms not only persisted in the ticks for a period of more than 200 days, but they were also ejected with the coxal fluids during the feeding process of the ticks. Since the publication of these observations, transmission experiments with the same lots of ticks have been carried out. Both species of ticks were able to transmit the agent to Swiss mice on which they were placed for feeding, but the observation that *Past. tularensis* infection is harmful and ultimately fatal to the ticks could not be confirmed.

In view of these findings, it seemed probable that argasid ticks, especially those of the genus *Ornithodoros*, were of greater importance in the maintenance and distribution of tularemia in nature than is generally presumed. In order to obtain more evidence on the accuracy of this opinion, a series of experiments was conducted in which *Ornithodoros* ticks were infected with *Past. tularensis*. The ticks were tested for their ability to ingest, preserve and transmit the organisms. It is the purpose of this paper to present the results of these studies.

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We wish to thank Mr. N. J. Kramis for the photographic work.

MATERIAL AND METHODS

Ornithodoros moubata (Africa), *O. turicata*, *O. parkeri* and *O. hermsi* (Western U. S.), which

FIG. 1.—Section of midgut of *O. moubata*. Marked area shows epithelial cells distended with *Past. tularensis*. ×55.

FIG. 2.—High power magnification of area marked in figure 1. ×500.

FIG. 3.—*Past. tularensis* in the hemolymph of *O. turicata;* blood cell packed with organisms. ×750.

FIG. 4.—Salivary gland of *O. moubata*. See marked area under high power magnification in figure 5. ×25.

→

were reared at the Rocky Mountain Laboratory, were infected with strains Nevada 14 and Schu SD of *Past. tularensis*. Nevada 14 was isolated from a hare (*Lepus californicus*) in 1951, and has since been used in the laboratory for experimental studies; strain Schu SD was isolated from a finger ulcer in 1941. Both strains were of such virulence that one viable organism was sufficient to kill a white mouse by intraperitoneal inoculation.

One hundred and fifty *O. moubata*, 4th nymphal stage, 100 *O. parkeri*, 4th nymphal stage, 200 *O. turicata*, 2nd nymphal stage and approximately 200 *O. hermsi* in the second nymphal stage were infected by feeding on mice which previously were inoculated intraperitoneally with 0.2 ml of a suspension of strain Nevada 14, diluted from 10^{-6} to 10^{-7}. One hundred and fifteen *O. moubata* in the 4th nymphal stage were similarly infected with *Past. tularensis*, strain Schu SD. In all instances, bacteremia in the blood donors was confirmed by examination of tail blood smears stained by Giemsa's method, before the ticks were placed on the mice for feeding. After the ticks had engorged, they were placed in glass vials and held at room temperature in desiccator jars in which a relative humidity of 78 to 80% was maintained.

The development of *Past. tularensis* in the ticks was followed by various techniques. The question of whether the agent develops in the gut tissues only, or invades the body cavity and the tick organs, was clarified by examining the hemolymph of ticks during the first 20 days of infection. This fluid was obtained by amputating the distal portion of the legs. In addition, anal excretions and coxal fluids, obtained by irritating the ventral side of the ticks with forceps, were examined from time to time for the presence of tularemia organisms. Smears were prepared from these fluids, stained by Giemsa's method and examined microscopically. In many instances the fluids were also cultured in order to confirm microscopic observations. At varying intervals, after the infectious blood meal, serial sections were prepared from tick organs. The ticks were dissected* and their tissues fixed in neutral buffered 10% formalin. Paraffin sections, 3 to 5 μ thick, were stained with buffered azure-eosinate after the method of Lillie (1948). In order to test the ability of the ticks to transmit *Past. tularensis* during their feedings, ticks were fed individually or in lots of 2 to 5 on normal Swiss mice.

RESULTS

The development of *Past. tularensis* in the ticks was followed for 450 days. The data obtained with the different species of ticks were similar and, therefore, are summarized together. With the infectious blood the bacteria arrive in the diverticula of the midgut where they invade the ameboic epithelial cells of the gut wall. In the cytoplasm of these cells a multiplication of the organisms takes place, forming distended, swollen cells, which eventually rupture and discharge their contents into either the gut lumen or the body cavity (fig. 1 and 2).

Between infected cells, in which masses of organisms can be seen, there are always normal epithelial cells without any bacteria. The discharge of the organisms into the body cavity probably occurs during the first days of infection, as the hemolymph of numerous

* A detailed description and illustration of the anatomy of *O. moubata* was presented by Burgdorfer (1951).

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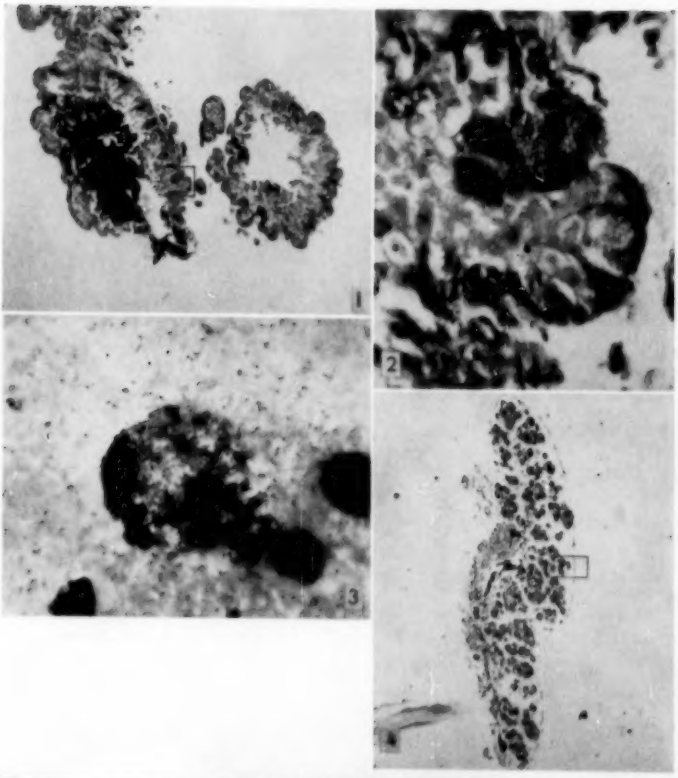
FIG. 5.—*Past. tularensis* in the tissue of a salivary gland of *O. moubata*. ×1000.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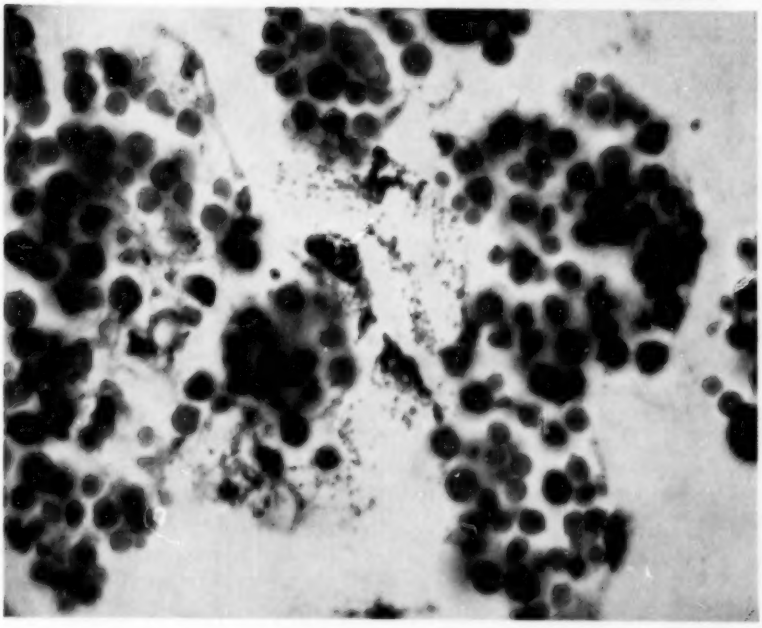

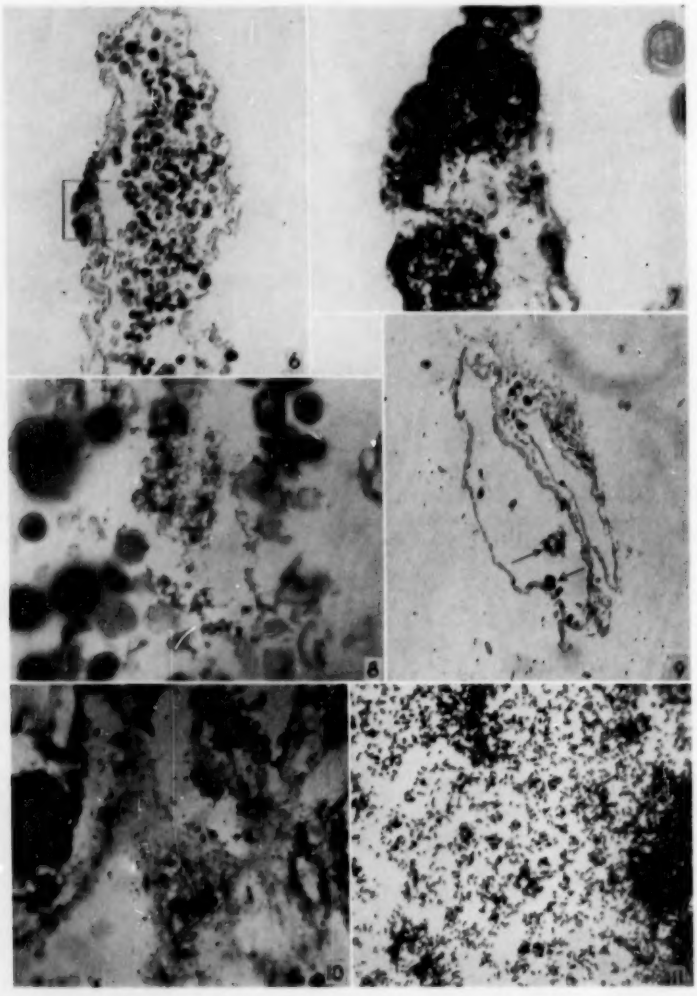

FIG. 6.—Section of Malpighian tubule of *O. moubata* showing *Past. tularensis* in the epithelium. ×120.

FIG. 7.—Area marked in figure 6 seen under high power magnification. ×500.

FIG. 8.—*Past. tularensis* in the anal excretions of *O. moubata*. ×500.

FIG. 9.—Cross section through the tubular system of the coxal organ of *O. moubata*. Masses of tularemia organisms are seen in the lumen as well as in the walls of the tubules. ×55.

FIG. 10.—Section through the filtration chamber of the coxal organ of *O. moubata* showing *Past. tularensis*. ×500.

FIG. 11.—Smear of infectious coxal fluid of *O. parkeri*. ×750.

TABLE 1.—*Presence of* Past. tularensis *in the coxal fluids of* O. moubata, O. turicata *and* O. parkeri *after ingestion of an infective blood meal.*

Species of ticks	Strain of *Past. tularensis*	Days after infectious feeding	Number of ticks tested	Positive coxal fluids	
				Number	Percent
O. moubata	Schu SD	10	50	0	0
		54	30	12	40
		62	65	18	28
		119	50	36	72
		210	50	34	68
O. moubata	Nevada 14	15	50	1	2
		42	50	6	12
		112	30	9	30
		221	29	10	34
O. parkeri	Nevada 14	20	50	6	12
		42	50	13	26
		100	30	12	40
		165	30	16	53
O. turicata	Nevada 14	49	100	23	23
		56	100	28	28
		100	160	52	39
		224	138	36	26

ticks showed masses of *Past. tularensis* as early as 10 days after the ticks had engorged upon the infected mice (fig. 3). No evidence could be found that the tissues of the gut wall became free of these bacteria with increased duration of infection: distended epithelial cells, packed with morphoolgically typical bacilli, were still present in the gut tissue, even after several feedings of the ticks on normal mice.

Anal excretions or "feces" of *O. turicata*, *O. parkeri* and *O. hermsi* were found to contain *Past. tularensis* as early as 30 days after the ticks ingested the infectious mouse blood. They proved to be infectious during the entire period of study. In the case of *O. moubata*, the anal excretions cannot be considered as feces, since the midgut of this tick is actually a blind sac, without connection to the hind gut or rectal ampules. The anal products are excretions from the rectal ampules, and the latter are entered only by the Malpighian tubules.

Following the discharge of *Past. tularensis* into the body cavity, an invasion of the salivary glands (fig. 4 and 5), central ganglion, coxal organs and tissues of the genital,* as well as excretory systems takes place. The epithelial cells of the Malpighian tubules and rectal ampules, especially, are packed with bacteria, seen in serial sections as blue-stained areas (fig. 6 and 7), which are easily detectable under the low power microscope. Bacilli were also found in the lumen of the excretory system; therefore, it was not surprising to find that the anal excretions of ticks, especially of *O. moubata*, contained masses of organisms (fig. 8). A very heavy concentration of bacteria was observed in the tissues of the coxal organs, which consist of a filtration chamber, an accessory gland and a system of tubules which leads to the external opening. The findings of organisms in the walls and lumen of these tubules (fig. 9 and 10), suggested that the agent was present also in the ejected coxal fluids. Therefore, at different intervals after the ticks ingested the infectious blood, the coxal fluids of *O. moubata*, *O. turicata* and *O. parkeri* were examined microscopically. The results are summarized in table 1. Only the fluids of ticks which ejected large masses of tularemia bacilli, as shown,

* A detailed description of the infection of the genital organs will be presented in a separate paper on ovarial transmission of *Past. tularensis* in tick vectors.

for instance, in figure 11, are reported as positive. The actual proportion of ticks ejecting infectious coxal fluids must be considered much higher, as small numbers of organisms are not detectable by routine microscopic examination.

TRANSMISSION EXPERIMENTS

Theoretically, *O. moubata*, *O. turicata*, and *O. parkeri* could transmit *Past. tularensis* by (a) bite, i.e. by injecting infectious saliva, (b) contamination of the bite wounds with infectious coxal fluids ejected shortly before the ticks finish feeding, and (c) contamination of the bite wounds with infectious anal excretions. In the case of *O. hermsi*, the second possibility must be eliminated as this tick's coxal fluids dry out as soon as they appear on the external openings of the coxal organs, where they are usually found in the form of white crystalline deposits.

Since *Past. tularensis* is present in the tissues of the salivary glands, in the ejected coxal fluids and in the anal excretions of the ticks, transmissions during the feedings could be expected. A total of 472 transmission experiments was performed at different intervals after the ticks ingested the infectious blood. Ticks were fed individually or in lots of 2 to 5 per mouse. The results, summarized in table 2, show that all species under study were able to transmit the agent during their feedings, but that the African tick, *O. moubata*, was the most efficient vector. It is probable that transmissions occurred by contamination of the bite wounds with infectious coxal fluids as well as directly by bite, i.e., by infectious saliva. However, the fact that *O. hermsi*, without liquid coxal fluids, was the poorest transmitter suggests that coxal fluids were of greater importance. Infections by contamination of the bite wounds with anal excretions were highly improbable, as ejection of the anal products usually takes place after the ticks leave their hosts.

DISCUSSION

The argasid ticks *O. moubata*, *O. turicata*, *O. parkeri* and *O. hermsi* have

TABLE 2.—*Experimental transmission of* Past. tularensis *from infected to normal mice by* O. moubata, O. turicata, O. parkeri *and* O. hermsi.

Species of ticks	Strain of *Past. tularensis*	Days after infectious meal	Number of ticks per mouse	Number of mice	Transmissions*	
					Number	Percent
O. moubata	Schu SD	29	5	20	3	15
		54	3	10	3	30
		62	5	13	7	54
		117	1	16	5	31
		119	3–5	10	9	90
		224	1–3	7	2	29
O. moubata	Nevada 14	43	5	21	9	43
		70	3–5	20	14	70
		112	1	30	4	13
		115	5	10	11	91
		220	1–3	9	7	78
		221	1	20	2	10
O. parkeri	Nevada 14	42	5	15	4	27
		97	3–5	14	3	21
		223	2	5	1	20
O. turicata	Nevada 14	29	5	45	0	0
		56	5	40	3	8
		100	3–5	40	5	13
		224	3–5	31	7	23
O. hermsi	Nevada 14	38	5	36	4	11
		71	5	20	1	5
		186	3–5	20	0	0
		226	2–5	20	0	0

* Transmission confirmed in each case by the recovery of *Past. tularensis* from the spleen by culture.

been found to be possible vectors of tularemia, in that they were able not only to preserve ingested *Past. tularensis* organisms for a period of longer than 450 days, but also to transmit the agent during feedings. These observations are in partial disagreement with those made by Davis (1940), who found that infected *O. turicata* and *O. parkeri* preserved but did not transmit the organisms. Kamil and Bilal (1938) obtained results similar to those reported here with *O. lahorensis*.

It is surprising that argasid ticks have not been incriminated in the transmission of tularemia nor ever found to be naturally infected with *Past. tularensis*. There are no records, however, of attempts to isolate the agent from argasid ticks collected in areas where tularemia is prevalent. Biological differences between the known vectors of the disease and the argasid ticks studied in this paper may explain why the latter have never been found to be involved in the epidemiological picture of the disease. With the exception of *O. moubata* of Africa, where tularemia has not as yet been recognized, the ticks *O. parkeri*, *O. turicata* and *O. hermsi* are very seldom found to parasitize man. Their blood donors are mainly small rodents (prairie dogs, jackrabbits, cottontail rabbits, mice, ground squirrels, tree squirrels, chipmunks, weasels, etc.) and birds. Cattle and chickens also have been reported to serve as hosts of *O. turicata*. The ticks engorge rapidly (1 to 30 minutes), and their feedings generally take place in the burrows and nests of the blood donors. As soon as they are engorged they drop to the ground and burrow into the soil, or in case of *O. hermsi*, which is parasitic chiefly on chipmunks and tree squirrels, they retreat into cracks and crevices of trees.

The ixodid ticks, on the other hand, parasitize a much wider range of hosts. Larvae and nymphs feed primarily on small rodents, adults also on larger wild as well as domestic animals, with which man is directly or indirectly in contact. In contrast with argasid ticks, they are characterized by long feeding periods (several days), during which they may be carried and distributed widely.

The ticks are easily collected either from their hosts or from the vegetation of an infested area.

SUMMARY

1. The argasid ticks *Ornithodoros moubata*, *O. turicata*, *O. parkeri*, and *O. hermsi* were infected with *Pasteurella tularensis* in order to test their ability to preserve and transmit this agent.

2. The course of infection in the ticks was followed over a period of 450 days. It was found that the ingested organisms developed and persisted in the tissues of the intestinal tracts, salivary glands, coxal organs, central ganglion, excretory and genital systems of all species of ticks studied.

3. The coxal fluids of *O. moubata*, *O. turicata*, and *O. parkeri*, as well as anal excretions of all four species were infectious in that they contained large masses of *Past. tularensis*.

4. A total of 472 transmission experiments in which the ticks were fed individually or in lots of 2 to 5 per mouse was performed. All species under study were able to transmit *Past. tularensis*. The most efficient vector was *O. moubata*.

5. The results presented in this paper suggest the possibility that argasid ticks may be of importance in the maintenance and distribution of *Past. tularensis* among their hosts in nature.

REFERENCES

Burgdorfer, W. 1951, Analyse des Infektionsverlaufes bei *Ornithodorus moubata* (Murray) und

der natuerlichen Uebertragung von *Spirochaeta duttoni*. Acta Trop. **8**: 193–262.

Burgdorfer, W. and Pickens, E. G. 1954, A technique employing embryonated chicken eggs for the infection of argasid ticks with *Coxiella burnetii*, *Bacterium tularense*, *Leptospira icterohaemorrhagiae*, and Western equine encephalitis virus. J. Infect. Dis. **94**: 84–89.

Burroughs, A. L. et al. 1945, A field study of latent tularemia in rodents with a list of all known naturally infected vertebrates. J. Infect. Dis. **76**: 115–119.

Davis, G. E. 1940, *Bacterium tularense*: Its persistence in the tissues of the argasid ticks *Ornithodoros turicata* and *O. parkeri*. Pub. Health Rep. **55**: 676–680.

———. 1943, Studies on the biology of the argasid tick, *Ornithodoros nicollei* Mooser. J. Parasitol. **29**: 393–395.

———. 1945, in Manual of Tropical Medicine, ed. I, Philadelphia & London, W. B. Saunders Co., section X, pp. 525–530.

Hopla, C. E. 1953, Experimental studies on tick transmission of tularemia organisms. Am. J. Hyg. **58**: 101–118.

Kamil, S. and Bilal, S. 1938, Recherches expérimentales sur l'étiologie de la tularémie en Turquie. Ann. Parasitol. humaine et comparée, XVI: 530–542.

Lillie, R. D. 1948, Histopathologic Technic, Philadelphia & Toronto, Blakiston Co.

Parker, R. R. et al. 1924, Tularemia infection in ticks of the species *Dermacentor andersoni* Stiles in the Bitterroot Valley, Montana. Pub. Health Rep. **39**: 1057–1073.

Steinhaus, E. A. 1946, Insect Microbiology, New York, Comstock Publishing Co. Inc.

THE RESTORATION OF HEMOLYSIN-FORMING CAPACITY IN X-IRRADIATED RABBITS BY TISSUE AND YEAST PREPARATIONS

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In general, decreased antibody production follows exposure to a large dose of hard X irradiation administered one or more days prior to the injection of antigen (review by W. H. and L. G. Taliaferro, 195[illegible]. W. H. and L. G. Taliaferro (195[illegible], studied radiation dose response in rabbits receiving 1.25 ml of a 1% standardized suspension of sheep red cells 2 days after irradiation and found that the ED_{50} for hemolysin formation in rabbits was approximately 240 r and the ED_{95} was 400 r. Protection of the primary antibody response in animals exposed to radiation ($\geqq ED_{95}$) has been reported by several workers. Jacobson et al (1952) exposed spleen-shielded rabbits to 800 r, splenectomized them 1 day later and the next day injected sheep red cells. Their results indicated that in these animals hemolysin formation was higher than in unshielded, irradiated controls. Wissler et al (1953) found that irradiated rats under similar conditions produced hemolysin when the red cells were injected intraperitoneally but not when they were injected intravenously. T. N. Harris et al (1954 and 1955) and S. and T. N. Harris (1955) induced antibody formation in X-irradiated (425 r) rabbits by injecting washed lymph node cells previously incubated with dysentery bacteria or their soluble antigens or by injecting cells and bacteria separately. Roberts and Dixon (1955), using bovine γ globulin in the same manner, i.e., with washed lymph node cells, detected no antibody response.

In the present work, we have studied the production of hemolysin to sheep red cells in total body X-irradiated (400 r) rabbits that received an intravenous injection of a mixture of antigen and various preparations 1 day after irradiation. The 1-day interval was selected because W. H. and L. G. Taliaferro (1954b) found that it was associated with maximum depression of antibody and recovery was approximately logarithmic as the interval was increased (50% recovery time $\approx$ 4 days). Antibody titers in irradiated treated rabbits were significantly higher than those of the irradiated control group when a preparation of normal rabbit spleen, normal mouse spleen, yeast autolysate, or HeLa cells (human epithelioid carcinoma of the cervix grown in tissue culture) was mixed with antigen just prior to injection. Preparations from normal rabbit kidney or normal rabbit muscle were ineffective. A preliminary account of this work has been reported by Jaroslow and Taliaferro (1955).

MATERIALS AND METHODS

The rabbits were females and weighed 2 to 3.5 kg. They were supplied by Scientific Small

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Animal Laboratory and Farm at Des Plaines, Illinois. They were fed Rockland rabbit ration ad libitum.

Tissues were suspended in aqueous 0.8% phosphate buffer at pH 7.4 and were thoroughly ground in a Potter-Elvehjem mill at 4 C. The tissue mince consisted of the ground suspension filtered through glass wool to decrease the possibility of embolic accidents upon administration. The tissue extract was the supernatant fluid after the ground suspension was centrifuged in an average gravitational field of 20,000 × g for ½ hour in a cold room. HeLa cells, obtained in tissue culture from National Microbiological Associates at Washington, D. C., were treated as above for the preparation of minces and extracts. Yeast autolysate was prepared as follows: brewers' yeast (supplied by Wahl-Henius Co., Chicago, Illinois) was washed, centrifuged, and pressed in a Carver press to a water content of about 75%. It was then driven through a coarse sieve, spread in a ½ inch layer on filter paper and dried at room temperature. This dried yeast was mixed 1:4 with phosphate buffer at pH 7.4 as needed, was allowed to stand overnight at room temperature and was centrifuged in the same manner as was the tissue mince. Either the mince or the supernatant extract was then mixed with antigen and allowed to stand at room temperature for 3 or more minutes (usually 10 minutes) prior to injection into the marginal vein of the rabbit's left ear.

All animals received a single intravenous injection of antigen. Most were administered approximately 1.6×10^9 sheep red cells (1 ml of 10%), but a few received 2×10^8 heated stromata (≈1.25 ml of 1% red cells). The suspensions of cells were standardized by photometric determinations of the amount of hemoglobin released by distilled water lysis (W. H. and L. G. Taliaferro, 1950). A 1% suspension is described below. The stromata were prepared by suspending the cells in isotonic phosphate buffered 0.85% saline, lysing gradually by resuspending successively in half and then quarter strength buffered saline and reversing the steps to full strength buffered saline. This procedure was repeated until all visible red color was removed from the suspension. The stromata were counted in a hemacytometer chamber with a phase contrast microscope and then autoclaved at about 2 lb pressure (approximately 103 C) for 1 hour.

A detailed description of the materials and methods used for the in vitro hemolysin titrations is to be found in the papers by W. H. and L. G. Taliaferro (1950) and Taliaferro et al (1952). Here, we will note a few salient points.

The hemolysin unit is that amount of serum which hemolyzes 50% of 2 ml of a standardized 1% suspension of sheep red cells, in the presence of four 50% units of complement, and titers are shown either as the amount of serum containing 1 50% unit or as the number of 50% units per ml. A 1% suspension of red cells (approximately 1.6×10^8 cells/ml) is one which when diluted 1:5 with distilled water gives a reading of 150 on the Klett Summerson photoelectric colorimeter with a no. 54 filter (spectral range 500 to 570 mμ).

The curve of hemolysin production was graphed on a semilogarithmic plot of the titers against time. The parameters of the immune process were log peak titer, length of the induction period and rate of accumulation (k_1) of antibody shown as a polyphase curve wherein the initial rate and average rate (derived k_1) of rise to peak titer were noted.

For the statistical analysis of the results, log peak titers were used because of the nature of the antibody response and to obviate the skewing of the distributions with arithmetic peak titers, while rates and times were treated arithmetically (see W. H. and L. G. Taliaferro, 1954a).

Radiation factors were 250 kv, 15 ma, with 0.5 mm Cu and 3.0 mm bakelite filters. The half-layer value was 1.5 mm Cu. Target distance was 76 cm and the dose rate was 29 r/min. All irradiated rabbits received 400 r whole body irradiation. The rabbits were enclosed in perforated aluminum boxes and irradiated by a horizontal beam in such a manner that each side received half the dose.

RESULTS

Tissue or cellular preparations of diverse origin were mixed with antigen prior to intravenous injection into rabbits irradiated 1 day previously with 400 r. Some enhanced the antibody response while others had no apparent effect. The data, compiled in table 1, indicate that restorative potency is not restricted to what are generally considered antibody-forming cells and/or tissues.

Mean log peak titer was used as the best single measure of antibody formation (W. H. and L. G. Taliaferro, 1952 and 1954). Recipients of mince or extract of normal unimmunized rabbit

spleen (0.4 to 1.6 g)* or extract of normal unimmunized mouse spleen (0.3 g) mixed with antigen produced antibody titers significantly higher than those in irradiated and lower than those in unirradiated controls. Mince or extract of HeLa cells (0.2 to 0.8 g) or yeast autolysate (1.8 g) resulted in titers comparable to the unirradiated controls and significantly higher than the irradiated controls. Administration of mince or extract of normal rabbit kidney (0.4 to 1.5 g) or mince or extract of normal rabbit muscle (0.9 to 2.1 g) resulted in titers at the level of the irradiated controls. The separate injection of mince or extract of normal rabbit spleen (1.0 to 2.7 g) 1 hour after irradiation and 1 or 2 days before antigen injection did not result in an elevated antibody titer. Smith and Ruth (1955) also reported that the injection of spleen mince into irradiated mice 1 day before sheep red cells did not result in an increased hemolysin response.

The mean log peak antibody titer obtained in the treated groups was positively correlated with the initial rate of antibody rise, but was not as highly correlated with the derived rate—possibly because of the comparatively inaccurate estimation of the time of peak titer. The consistently lower derived k_1 rate and the consistently longer rise to peak titer in the irradiated rabbits as compared to the unirradiated controls indicate a slower production of antibody even when normal amounts were formed. The induction period differed significantly from unirradiated controls ($P \leq 0.0005$).

The antibody curves for three rabbits typical for each of the three groups, are shown in figure 1. The differences and

TABLE 1.—*Hemolysin response in 6 groups (4 through 9) of rabbits receiving a mixture of a standardized suspension of red blood cells or stromata and various tissue minces or extracts 24 hours after exposure to 400 r total-body irradiation. There are 3 control groups (1 through 3).**

	Mean peak titer			Rate of accumulation (k_1)		Length in days of:		
	Log‡	Geometric X10[6]	Arithmetic X10[6]	First phase‡	Derived when > one phase‡	Induction period‡	Antibody rise to peak‡	Number of rabbits†
1 No X ray	3.28 ± .06 a	1.90	3.03 ± .46	1.1 ± .04 a	0.8 ± .04 a	2.6 ± 0.2 a	6.1 ± 0.3 a	51
2 X ray only	2.35 ± .10 A	0.22	0.45 ± .09	0.1 ± .06 A	0.4 ± .04 A	7.1 ± 0.2 A	9.8 ± 0.9 A	26 (8)
3 Rabbit spleen before antigen	2.11 ± .44 A	0.13	0.21 ± .05	0.8 ± .15 B	0.5 ± .09 B	7.6 ± 0.5 A	8.0 ± 1.7 A	8 (5)
4 Rabbit kidney	2.37 ± .10 A	0.24	0.33 ± .08	0.9 ± .08 C	0.4 ± .06 A	7.5 ± 1.6 A	11.2 ± 1.8 A	13 (11)
5 Rabbit muscle	2.25 ± .11 A	0.18	0.35 ± .08	0.9 ± .08 B	0.4 ± .04 A	6.3 ± 0.2 A	8.1 ± 0.6 A	12 (10)
6 Rabbit spleen	2.70 ± .06 Ab	0.50	0.92 ± .15	1.0 ± .06 Cb	0.5 ± .04 A	6.9 ± 0.3 A	9.3 ± 0.7 A	37 (8)
7 Mouse spleen	2.92 ± .13 Cb	0.84	1.16 ± .35	1.3 ± .22 Ca	0.7 ± .14 b	6.1 ± 0.1 A	8.1 ± 1.5 A	8
8 HeLa cells	3.26 ± .14 a	1.80	2.30 ± .54	1.1 ± .14 b	0.6 ± .07 b	6.7 ± 0.3 A	9.8 ± 1.3 A	9
9 Yeast autolysate	3.17 ± .23 a	1.50	2.35 ± .59	1.1 ± .08 b	0.5 ± .01 B	6.3 ± 0.3 A	10.3 ± 0.4 A	6 (1)

* In group 3 a preparation of normal rabbit spleen was injected within 1 hour after exposure to X rays and antigen was injected a day later. In groups 4 through 9 a mixture of antigen and a specific preparation were injected a day after exposure to X rays. Groups 3 through 9 consist of all the animals receiving the particular preparation irrespective of whether it was in the form of mince or extract. Group 1 includes animals receiving an antigen-tissue mixture or antigen only.

† Numbers in parentheses refer to additional animals that gave no antibody response. Mean peak titers were calculated from entire groups, whereas the other measurements show the mean ± the standard errors for the antibody producers only.

‡ Data were compared to unirradiated controls when capital letters are used and to irradiated controls when lower case letters are used. When no letter occurs, $P > 0.05$. A or a, $P \leq 0.001$. B or b, $P = 0.002$ to 0.01. C or c, $P = 0.02$ to 0.05. Probabilities were not calculated for arithmetic mean peak titers.

* Yeast after air drying and fresh tissues were weighed before processing. Wet weights of packed HeLa cells were approximated from volumetric determinations.

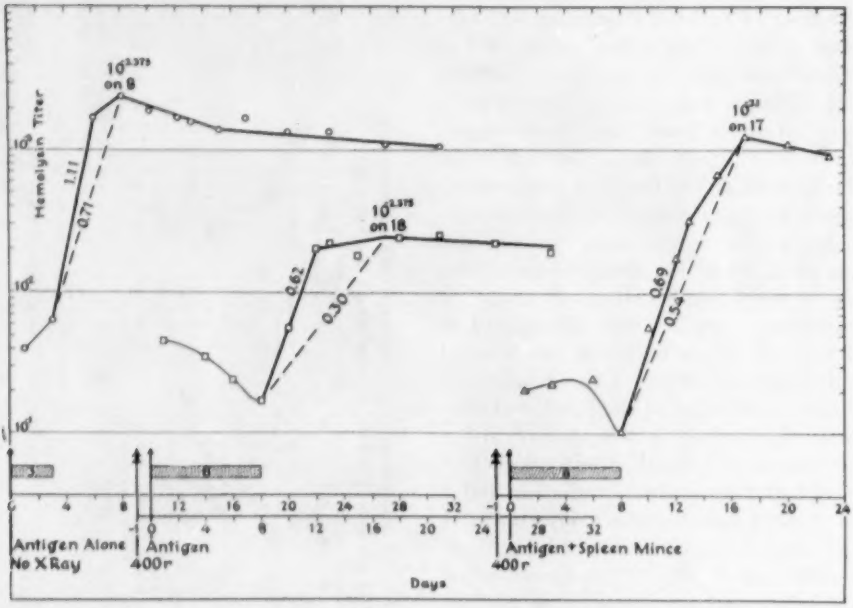

FIG. 1.—Antibody curves from 3 rabbits, typical of the groups to which they belong. Note that the irradiated rabbit receiving spleen mince showed a peak titer and rate of antibody accumulation intermediate between those in the unirradiated and irradiated controls and a lengthened induction period similar to that in the irradiated control.

similarities of the characteristics discussed earlier are remarkably well demonstrated except for initial k_1. For better visualization of the differences in mean log peak titers, figure 2 depicts the distribution of the individual log peak titers for each treated group and controls.

In calculating mean rates and times, the rabbits giving no antibody response were omitted since the assignment of arbitrary values would have resulted in a skewed distribution. In calculating the mean log titer, on the other hand, the log peak titer of such animals was considered to be the pre-immunization titer.

No significant correlation was noted between log peak titer and amount of tissue injected or the time of tissue-antigen contact (3 to 90 minutes) prior to injection.

Within the groups, no significant difference was observed between animals receiving tissue extract and those receiving tissue mince. Similarly, no significant difference was noted between animals that received stromata and those that received whole red cells (compare distribution in figure 2). Therefore, these data were combined for the calculation of each group mean.

The difference in peak titer was not statistically significant ($P>0.10$) between unirradiated animals which rereceived a tissue-antigen mixture and those which received only antigen. These groups were combined into one group labelled "antigen control" in figure 2 and "group 1" in table 1. The

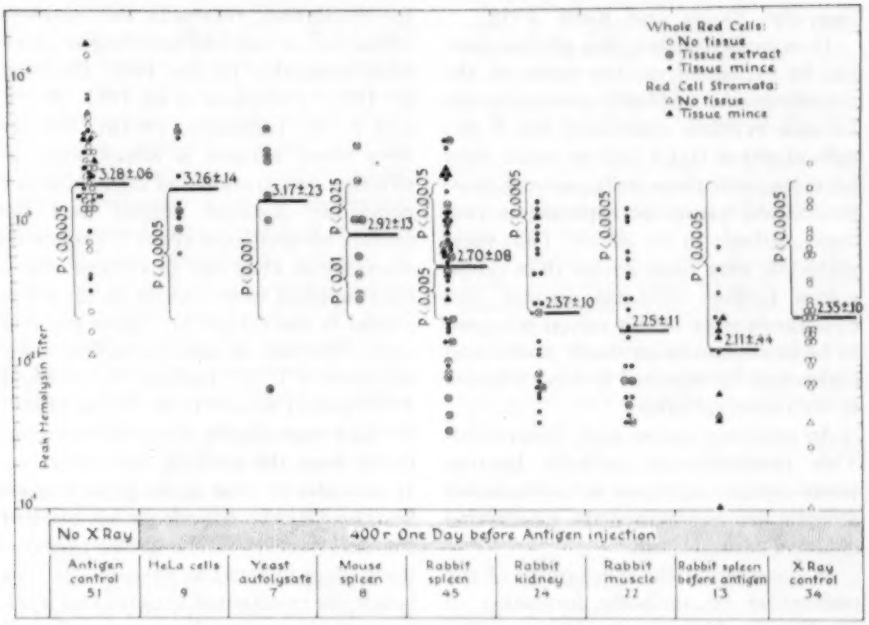

FIG. 2.—Log peak antibody titers in 7 treated groups and 2 control groups. Probabilities (t test) are indicated for the differences between each treated group and the antigen control group (extreme left) and X-ray control group (extreme right). Where no probabilities are shown $P > 0.05$.

only change in P values that resulted from this combination was in the comparison with mouse spleen ($P < 0.05$ to $P < 0.025$) in figure 2.

DISCUSSION

As compared to X-rayed (400 r) controls receiving antigen 1 day later, X-rayed rabbits produced more hemolysin when antigen was mixed with preparations of rabbit spleen, mouse spleen, HeLa cells, or yeast autolysate just prior to intravenous injection. The enhancement was most pronounced with materials from heterologous sources. Rabbit spleen or mouse spleen preparations produced marked effects, but only HeLa cells and yeast autolysate enabled the rabbits to produce antibody with peak titers statistically identical to unirradiated controls. The difference in potency among tissues is not understood and merits further investigation. Rabbit kidney or muscle preparations showed little or no activity. This finding may be associated with a concentration effect.

It has been observed that spleen-shielded irradiated rabbits (Jacobson et al, 1952) or rats (Wissler et al, 1953) produced higher antibody titers when they were splenectomized 1 day after irradiation and given sheep red cells 2 days after irradiation. The mechanism responsible for this phenomenon is probably not the one involved in the present study. If it were, rabbit spleen mince or extract injected shortly after irradiation and 1 day prior to antigen administration should result in increased hemolysin production in the irradiated recipients

(see also Smith and Ruth, 1955).

It is unlikely that this phenomenon can be explained on the basis of the transference of antibody-producing cells because extracts containing few if any cells of either HeLa cells or yeast, were effective, and these cells, even if present, would hardly be expected to produce antibody in the rabbit. That these materials were more active than rabbit spleen further militates against this hypothesis since rabbit spleen is known to be important in antibody production and would be expected to have a better in vivo survival value.

An adjuvant action may be involved. This possibility is unlikely because tissue-antigen mixtures in unirradiated animals did not induce the production of above normal titers.

In considering the mechanism of the restoration of antibody formation in irradiated rabbits, it should be emphasized that the capacity to form normal amounts of antibody was restored but only after a longer induction period and probably at a slower rate. In a real sense, mixing the antigen with HeLa cells or yeast preparations changed the immune response from the type usually seen in irradiated rabbits receiving antigen after irradiation to that encountered in rabbits receiving antigen before irradiation. Thus, W. H. and L. G. Taliaferro (1954b) found when antigen was given from 0.2 hours to 2 days before 500 r total body irradiation, the induction period was significantly lengthened and k_1 and derived k_1 rates decreased, whereas log mean peak titer attained the normal range. In fact, in the group receiving antigen 2 hours before irradiation, the mean log peak titer was increased ($P = 0.01$) above unirradiated controls.

Various workers have adduced evidence for a short radiosensitive stage in antibody formation which, if eliminated by irradiation, results in the complete inhibition or marked lowering of antibody formation (Kohn, 1951; Dixon et al, 1952; Taliaferro et al, 1952; W. H. and L. G. Taliaferro, 1954b). We believe when antigen is mixed with an effective preparation and injected into a previously X-rayed animal that the initial radiosensitive event (1) proceeds normally in vivo due to cells or materials supplied to the rabbit or (2) is bypassed in the rabbit, i.e., takes place in vitro. The first sequence is indicated by the work of T. N. Harris et al (1954 and 1955) and of S. and T. N. Harris (1955) in which washed cells, when injected separately from the antigen, were effective. It may also be true in the present work because we have not adequately studied the effectiveness of the active preparations when injected at various intervals before the injection of antigen. Our work suggests that restoration can be effected by materials in solution or in aggregates of subcellular organization. Whatever the mechanism, the treated animal forms normal amounts of antibody but at a slower rate (lengthened induction period and, at times, a slower rate of antibody rise). The slowing of the immune response is characteristic of the relatively radioresistant process seen in animals receiving antigen before irradiation. The restorative principle is apparently present in cells of different types and origin but not in sufficient concentration in all cells.

SUMMARY

Restoration of antibody-forming capacity in varying degrees as gauged by peak titer occurred in rabbits when injected a day after irradiation (400 r) with a mixture of sheep red cell antigen and one of several preparations, viz, normal unimmunized rabbit spleen mince or extract, normal mouse spleen extract, HeLa cell mince or extract, or

yeast autolysate. Essentially complete restoration was effected by HeLa cell mince or extract and yeast autolysate. Normal rabbit kidney mince or extract and normal rabbit muscle mince or extract were ineffective when injected similarly. In addition, normal rabbit spleen preparations were ineffective when injected into irradiated rabbits 24 or 48 hours prior to the injection of antigen. The induction period in all irradiated rabbits was longer (7 or more days) than in unirradiated animals (3 to 4 days) irrespective of whether the antibody-forming capacity was restored.

REFERENCES

Dixon, F. J., Talmage, D. W. and Maurer, P. H. 1952, Radiosensitive and radioresistant phases in the antibody response. J. Immunol. **68:** 693–700.

Harris, S. and Harris, T. N. 1955, Studies on the transfer of lymph node cells, V. Transfer of cells incubated in vitro with suspensions of *Shigella paradysenteriae*. J. Immunol. **74:** 318–328.

Harris, T. N., Harris, S. and Farber, M. B. 1954, Transfer to X-irradiated rabbits of lymph node cells incubated in vitro with *Shigella paradysenteriae*. Proc. Soc. Exper. Biol. & Med. **86:** 549–553.

———. 1955, Studies on the transfer of lymph node cells. VI. Transfer of cells incubated in vitro with *Shigella* treated serum. J. Immunol. **75:** 112–122.

Jacobson, L. O. and Robson, M. J. 1952, Factors effecting X-ray inhibition of antibody formation. J. Lab. & Clin. Med. **39:** 169–175.

Jaroslow, B. N. and Taliaferro, W. H. 1955, Hemolysin production in X-irradiated rabbits. Radiation Res. **3:** 237.

Kohn, H. I. 1951, Effect of X-rays upon hemolysin production in the rat. J. Immunol. **66:** 525–533.

Roberts, Jr., J. C. and Dixon, F. J. 1955, The transfer of lymph node cells in the study of the immune response to foreign proteins. J. Exper. Med. **102:** 379–392.

Smith, F. and Ruth, H. J. 1955, Hemolysin production in irradiated mice given spleen or bone-marrow homogenate. Proc. Soc. Exper. Biol. & Med. **90:** 187–191.

Taliaferro, W. H. and Taliaferro, L. G. 1950, The dynamics of hemolysin formation in intact and splenectomized rabbits. J. Infect. Dis. **87:** 37–62.

———. 1951, Effect of X-rays on immunity; a review. J. Immunol. **66:** 181–212.

———. 1952, The role of the spleen and the dynamics of hemolysin production in homologous anamnesis. J. Infect. Dis. **90:** 205–232.

———. 1954, Effect of X rays on hemolysin formation following various immunization and irradiation procedures. J. Infect. Dis. **95:** 117–133.

———. 1954b. Further studies on the radiosensitive stages in hemolysin formation. J. Infect. Dis. **95:** 134–141.

Taliaferro, W. H., Taliaferro, L. G., and Janssen, E. F. 1952, The localization of X-ray injury to the initial phases of antibody response. J. Infect. Dis. **91:** 105–124.

Wissler, R. W., Robson, M. J., Fitch, F., Nelson, W. and Jacobsen, L. O. 1953, The effects of spleen shielding and subsequent splenectomy upon antibody formation in rats receiving total-body X-irradiation. J. Immunol. **70:** 379–385.

DEMONSTRATION OF *SHIGELLA FLEXNERI* ANTIGENS BY MEANS OF HEMAGGLUTINATION TEST

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The principle of the indirect bacterial hemagglutination test is based upon the fact that certain bacterial antigens can be adsorbed on the red blood cells, thus rendering them specifically agglutinable by homologous bacterial antiserums. This test was first reported by Keogh et al[1,2] and used for the detection of antigens of various bacteria by many workers.[3–7]

The bacterial antigens that can be adsorbed on red blood cells are soluble components liberated from bacteria by various treatments, such as polysaccharides,[2,6,8] protein,[9] and 0 group antigens of *Escherichia coli*.[5,10]

The antigenic structure of *Shigella flexneri* is, because of crossagglutinating factors, the most complicated among the shigellas. Although recently classification by international agreement has been established,[11] there remain many problems unresolved concerning group antigens and variation of shigellas.

The agglutination and agglutinin absorption techniques are widely used for the detection of the antigenic composition of *Sh. flexneri*. However, these techniques are not applicable to determination of antigenic factors dissolved in the culture medium, and precipitation and complement-fixation reactions are generally used for the detection of soluble antigens of bacteria. If the soluble antigenic factors of *Sh. flexneri* can be determined easily and specifically by the indirect hemagglutination method, this procedure may offer advantages over the precipitation and complement-fixation tests.

The purpose of this study is to determine whether *Sh. flexneri* antigens can be demonstrated by indirect hemagglutination test and to evaluate the results

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1. Keogh, E. V., North, E. A. and Warburton, M. F. 1947, Hemagglutinins of the *Hemophilus* group. Nature, **160**: 63.
2. Keogh, E. V., North, E. A. and Warburton, M. F. 1948, Adsorption of bacterial polysaccharides to erythrocytes. Nature, **161**: 687–688.
3. Middlebrook, G., and Dubos, R. J. 1948, Specific serum agglutination of erythrocytes sensitized with extracts of tubercle bacilli. J. Exper. Med. **88**: 521–528.
4. Boyden, S. V. 1950, Adsorption by erythrocytes of antigens of *Pfeifferella mallei* and *Pfeifferella whitmori*. Proc. Soc. Exper. Biol. & Med. **73**: 289–291.
5. Neter, E., Bertram, L. F. and Arbesman, C. E. 1952, Demonstration of *Escherichia coli* 055 and 0111 antigens by means of hemagglutination. Proc. Soc. Exper. Biol. & Med. **79**: 255–257.
6. Wright, G. and Feinberg, R. J. 1952, Hemagglutination by Tularemia antisera: further observation on agglutination of polysaccharide-treated erythrocytes and its inhibition by polysaccharide. J. Immunol. **68**: 65–71.
7. Neter, E. Personal communication (*Shigella sonnei* hemagglutinins).
8. Alexander, N., Wright, G. and Baldwin, A. C. 1950. Observation on the agglutination of polysaccharide-treated erythrocytes by tularemia antisera. J. Exper. Med. **91**: 561–566.
9. Boyden, S. V. 1951, The adsorption of proteins on erythrocytes treated with tannic acid and subsequent hemagglutination by antiprotein sera. J. Exper. Med. **93**: 107–120.
10. Neter, E., Gorzynski, E. A., Zalewski, N. J., Rachman, R. and Gino, R. M. 1954, Studies on bacterial hemagglutination. Am. J. Pub. Health, **44**: 49–54.

in comparison to those of routine agglutination and complement-fixation tests.

MATERIAL AND METHODS

Fourteen type strains of *Sh. flexneri* 1 to 6, kindly supplied by Dr. W. H. Ewing, Communicable Disease Center, Atlanta, Georgia, were used as the test organisms.

The organisms were maintained on nutrient agar (composed of 1% Bacto-peptone, 0.3% sodium chloride, 0.5% beef extract, and 2% agar at pH 7.0), and smooth colonies, selected by using trypaflavin solution (0.2%), were used for the experiments.

For the hemagglutination test, 18- to 24-hour cultures of each type strain on nutrient agar or in broth were employed.

The antiserums were produced in rabbits by injection of suspensions of the type strains; the type-specific and group factor serums were prepared, as described by Edwards et al,[11] by absorption of the antiserums with suitable type strains.

The red blood cells for the hemagglutination test were washed in physiological saline three to four times, and the sediment of the cells was added, for modification, to the supernatant fluid of bacterial suspension or broth culture. After 2 hours of incubation at 37 C with repeated shaking the cells were washed three times with physiological saline and then resuspended in saline equal to the original volume. The treated cell suspension (0.25 ml) was added to 0.25 ml of immune serums in serial dilutions and to normal serum for control purposes.

The results were tentatively read after 2 hours of incubation in a water bath at 37 C and again after 18 to 24 hours at room temperature, according to the method described by Salk.[12]

The complement-fixation reaction was carried out by means of a modified procedure of Kolmer's method.[13]

RESULTS

First studied was the question as to whether *Sh. flexneri* antigens modify red blood cells, and, if so, what effect the concentration of the antigen has upon this reaction. Suspensions of agar and broth cultures of *Sh. flexneri* 1*a* were treated at 100 C for 1 hour and centrifuged. Washed goat red blood cells were added for modification to these supernates in various concentrations to make 2.5% suspensions. The results, as shown in table 1, show that the greater

11. Edwards, P. R. and Ewing, W. H. 1951, A manual for enteric bacteriology. Public Health Service, Georgia.

12. Salk, J. E. 1944, A simplified procedure for titrating hemagglutination capacity of influenza virus and the corresponding antibody. J. Immunol. **49**: 87–98.

13. Kolmer, J. A., Spaulding, E. H. and Robinson, H. W. 1951, Approved laboratory technic. ed. 5, New York, Appleton-Century-Crofts, Inc.

TABLE 1.—*The effects of bacterial concentrations of* Sh. flexneri 1*a on the modification of goat red cells.*

			Hemagglutination							
	Concentration	Serum *Sh. flexneri*	Dilution							
			40	80	160	320	640	1280	2560	5120
Plate culture	2 plates*	1a	4†	4	4	4	4	2	—	—
		2b	3	1	—	—	—	—	—	—
	1 plate	1a	4	4	4	4	4	4	2	—
		2b	3	1	—	—	—	—	—	—
	½ plate	1a	4	4	4	4	4	4	3	—
		2b	4	3	1	—	—	—	—	—
	¼ plate	1a	4	4	4	4	4	3	—	—
		2b	4	4	3	2	—	—	—	—
	⅛ plate	1a	4	4	4	4	4	4	2	—
		2b	4	4	3	1	—	—	—	—
Broth culture	1 day culture	1a	—	—	—	—	—	—	—	—
		2b	—	—	—	—	—	—	—	—
	5 day culture	1a	4	4	4	2	1	—	—	—
		2b	—	—	—	—	—	—	—	—

* Indicated plate cultures were suspended in 10 ml of saline and boiled at 100 C 1 hour.
† 4, 3, 2, and 1 indicate the degrees of agglutination. —, negative reaction.

TABLE 2.—*The effects of the concentrations of red cells on the modification by the boiled supernatant of* Sh. flexneri 1*a suspension (one plate culture in 10 ml of saline).*

Concentration of red cells to be modified	Serum *Sh. flexneri*	Hemagglutination Dilution 40	80	160	320	640	1280	2560	5120
5%	1a	4	4	4	4	3	2	—	—
	2b-	2	—	—	—	—	—	—	—
2.5%	1a	4	4	4	4	4	4	2	—
	2b-	3	2	—	—	—	—	—	—
1.25%	1a	4	4	4	4	4	4	3	1
	2b-	4	3	2	1	—	—	—	—
0.625%	1a	4	4	4	4	4	4	4	3
	2b-	4	4	4	3	2	—	—	—

the concentration of antigen, the more pronounced is the modifying effect resulting in hemagglutination with homologous serum in high titer and with heterologous serum in low titer only. The growth from one agar plate (standard Petri dish with diameter of 10 cm was used) suspended in 10 ml of saline was best for modification of red cells under the present experimental conditions. The broth cultures were less effective than the bacterial suspensions of agar cultures.

To determine the effect of concentration of red blood cells, washed goat red cells were added to the supernatant fluid of boiled bacterial suspension (one agar plate culture in 10 ml of saline) so as to make respectively, 5.0, 2.5, 1.25, and 0.625% erythrocyte suspensions. As shown in table 2, the red cell suspension of higher concentration shows more specific modification than that of lower concentration. However, the titer of serum causing hemagglutination with the red cells of higher concentration is slightly lower than that with cells of lower concentration. Moreover, with red cells of a concentration below 1.25% it is difficult to determine the end point of positivity because of the vagueness of the reaction. In this experiment, the best result was obtained when red cells were used in concentration of 2.5%.

Red blood cells of man (O and AB type as well as Rh positive), rabbit, and guinea pig were equally suitable for modification as the goat erythrocytes.

The next series of experiments was carried out to compare the modifying activity of bacterial suspension treated by various ways with that of the suspensions boiled for 1 hour. One agar plate culture of *Sh. flexneri* 1*a* suspended in various buffers ranging from pH 2.0 to pH 10.0, and in isotonic saline were treated at 100 C, 52 C, and 37 C for various lengths of time. After centrifugation, the pH of the supernates was adjusted to 7.0 with sodium hydroxide or hydrochloric acid. Washed goat red blood cells (2.5%) were modified with these materials and tested as described above. The data obtained in this experiment are summarized in table 3. It can be clearly seen that the supernates of suspensions boiled at pH 6.0, 7.0, and 10.0 modify the red blood cells excellently, resulting in specific hemagglutination with homologous antiserums in high titer and in relatively weak cross-reaction with *S. flexneri 2a* serum. The suspension treated at 52 C for 5 days at pH 7.0 also modifies red cells excellently, but treatment at 52 C and 37 C for 1 day yields poor modifying antigen. Acid (below pH 5.0) treated antigen failed to modify the erythrocytes.

To determine the type specificity of the hemagglutination test, the follow-

Table 3.—*The effects of pH and heat on the red-cell-modifying activity of* Sh. flexneri 1a *suspension.*

pH	Heat treatment	Serum *Sh. flexneri*	Hemagglutination Dilution 40	80	160	320	640	1280	2560	5120
2.0	100 C 1 hr	1a	—	—	—	—	—	—	—	—
		2b	—	—	—	—	—	—	—	—
	52 C 5 days	1a	—	—	—	—	—	—	—	—
		2b	—	—	—	—	—	—	—	—
	37 C 1 day	1a	—	—	—	—	—	—	—	—
		2b	—	—	—	—	—	—	—	—
3.0	100 C 1 hr	1a	—	—	—	—	—	—	—	—
		2b	—	—	—	—	—	—	—	—
4.0	100 C 1 hr	1a	—	—	—	—	—	—	—	—
		2b	—	—	—	—	—	—	—	—
5.0	100 C 1 hr	1a	2	—	—	—	—	—	—	—
		2b	—	—	—	—	—	—	—	—
6.0	100 C 1 hr	1a	4	4	4	4	4	3	1	—
		2b	3	2	—	—	—	—	—	—
7.0	100 C 1 hr	1a	4	4	4	4	4	4	2	—
		2b	4	2	—	—	—	—	—	—
	52 C 5 days	1a	4	4	4	4	4	3	1	—
		2b	4	3	—	—	—	—	—	—
	52 C 1 day	1a	4	3	1	—	—	—	—	—
		2b	—	—	—	—	—	—	—	—
	37 C 1 day	1a	1	1	—	—	—	—	—	—
		2b	—	—	—	—	—	—	—	—
10.0	100 C 1 hr	1a	4	4	4	4	4	4	2	—
		2b	4	2	—	—	—	—	—	—
Saline	100 C 1 hr	1a	4	4	4	4	4	4	2	—
		2b	3	2	—	—	—	—	—	—

ing experiment was carried out. Erythrocytes were modified with supernates of boiled suspensions of various types (the culture of one agar plate in 10 ml of isotonic saline solution) and mixed with equal amounts (0.25 ml) of type specific and group factor *Sh. flexneri* antiserums in serial dilution. The resulting hemagglutination was read, and the data are presented in table 4.

It is evident from the table that in most instances the serums agglutinate to high titer red blood cells treated with homologous bacterial suspension and only in relatively low titer those treated with heterologous antigens. These re-

Table 4.—*The results of hemagglutination tests of* Sh. flexneri *with their antiserums.*

Strain	*Sh. flexneri* serum 1a	1b	2a	2b	X	Y	3(1)	4a	4b	5	6
Sh. flexneri											
1a	1280	80	160	80	160	40	—	40	40	80	—
1b	320	640	40	—	40	—	80	—	—	—	—
2a	—	—	640	320	320	320	—	—	—	—	—
2b	—	—	320	320	320	160	—	—	—	—	—
Var. X	—	—	—	—	—	—	—	—	—	—	—
Var. Y	—	—	640	320	1280	640	—	40	—	—	—
3(1)	—	640	80	—	1280	—	320	80	80	80	—
3(2)	—	320	—	40	2560	—	320	40	40	80	—
4	80	—	80	—	2560	80	—	160	160	320	—
4a	—	—	—	—	—	—	—	1280	320	40	—
4b	80	—	160	—	160	40	—	640	320	160	—
5	—	—	—	—	160	—	—	—	—	640	—
6(1)	—	—	—	—	—	—	—	—	—	—	160
6(2)	—	—	—	—	—	—	—	—	—	—	80

Titers are expressed as the reciprocals of the highest dilution of serum that gave hemagglutination.
— indicates the negative reaction at serum dilution 1:40.

TABLE 5.—*The results of hemagglutination tests and complement-fixation reactions of* Sh. flexneri *with type-specific serums.*

Serum	Hemagglutination						Complement-fixation					
	I	II	III	IV	V	VI	I	II	III	IV	V	VI
Sh. flexneri												
1a	640	—	—	—	—	—	1280	—	—	—	—	—
1b	160	—	—	—	—	—	640	—	—	—	—	—
2b	—	160	—	—	—	—	—	80	—	—	—	—
2b	—	160	—	—	—	—	—	80	—	—	—	—
Var. X	—	—	—	—	—	—	—	—	—	—	—	—
Var. V	—	—	—	—	—	—	—	—	—	—	—	—
3(1)	—	—	80	—	—	—	—	—	40	—	—	—
3(2)	—	—	160	—	—	—	—	—	40	—	—	—
4	—	—	—	320	—	—	—	—	—	320	—	—
4a	—	—	—	2560	—	—	—	—	—	640	—	—
4b	—	—	—	640	—	—	—	—	—	640	—	—
5	—	—	—	—	640	—	—	—	—	—	320	—
6(1)	—	—	—	—	—	80	—	—	—	—	—	80
6(2)	—	—	—	—	—	80	—	—	—	—	—	80

Titers of hemagglutination are expressed as the reciprocals of the highest dilution of serum that gave agglutination.
Titers of complement-fixation are expressed as the highest dilution of serum that gave complete inhibition of hemolysis.
These reactions are carried out by the method of Kolmer (the antiserums were used in successive dilution).
—, negative reaction at 1:40.

sults largely parallel those of the conventional bacterial agglutination test. It is impossible to determine the type or subtype by the hemagglutination method alone, as can be done with the bacterial agglutination technique. The results obtained with variant X strain are quite different. Red blood cells treated with this antigen are not agglutinated even by homologous antiserum, which readily agglutinates the bacterial cells. It is interesting to note that this serum in relatively high titer reacts with red blood cells modified with most other type strains.

The results obtained with type specific and group specific antiserums are listed in tables 5 and 6. The serums have been shown to agglutinate specifically the strains possessing the corresponding antigens. It can be seen from table 5 that similar results were obtained in hemagglutination and complement-fixation tests with absorbed type specific antiserums, and from table 6 that with absorbed group specific antiserums the results of hemagglutination test parallel those of complement fixation rather than those of bacterial agglutination tests.

DISCUSSION

The experiments described above revealed that the antigens of *Sh. flexneri* modify red blood cells of man, goat, rabbit, and guinea pig, the corresponding

TABLE 6.—*The results of agglutination, hemagglutination, and complement-fixation tests of* Sh. flexneri *with group-factor serums.*

Serum	Agglutination			Hemagglutination			Complement-fixation		
	(3), 4	6	7, (8)	(3), 4	6	7, (8)	(3), 4	6	7, (8)
Sh. flexneri									
1a	2560	—	—	—	—	—	80	—	—
1b	1280	5120	—	—	1280	—	—	320	—
2a	5120	—	—	640	—	—	640	—	—
2b	—	—	2560	—	—	80	—	—	80
Var. X	—	—	1280	—	—	—	—	—	—
Var. V	5120	—	—	80	—	—	160	—	—
3(1)	1280	5120	—	—	420	—	—	320	—
3(2)	—	5120	1280	—	160	—	—	160	—
4	640	—	—	—	—	—	—	—	—
4a	160	—	—	—	—	—	—	—	—
4b	320	—	—	—	—	—	—	—	—
5	320	—	—	—	—	—	—	—	—
6(1)	320	—	—	—	—	—	—	—	—
6(2)	320	—	—	—	—	—	—	—	—

antiserums causing indirect hemagglutination.

Indubitably, many factors effect the process of modification of red blood cells, but this report is concerned only with certain fundamental problems, such as the effects of heat, pH, concentration of bacterial antigen, and the kind of red blood cells.

It is unlikely that the red blood cells adsorb whole bacteria or antigenic substances normally present within the bacterial cell. Rather, antigens which are liberated from the bacterial cells by treatment and thus become dissolved in the diluent are adsorbed by erythrocytes. This assumption is supported by the observation that the hemagglutinating activity of supernates of boiled suspensions runs parallel to that of complement-fixing capacity rather than to agglutinibility of bacterial suspensions (table 6).

As shown by Neter et al[5] and in the present experiments, boiling of bacterial suspensions is an excellent method of treatment for the procurement of erythrocyte modifying antigens. Neter et al[5] at first considered the possibility that heat destroys an inhibitor, but we, as did Neter in a later communication,[10] consider it more likely that the effect of heat on bacterial suspension causes release of red blood cell modifying antigen.

Obviously, the hemagglutination test described in this communication does not lend itself to the study of heat labile antigens, since the latter are destroyed by heat treatment. In this connection our observation that treatment of bacteria at 52 C is effective in the release of erythrocyte modifying antigen may be used to advantage in further experiments on hemagglutination with heat labile antigens.

The negative results obtained with antigens treated in an acid medium can be explained by the assumption that the red blood cell modifying antigens are not extracted or are destroyed at low pH, particularly since the supernates of acid treated suspensions also prove to be antigenically inactive in complement-fixation tests.[14]

Further studies are needed to determine the reasons for the negative results obtained with variant X strain.

SUMMARY

A study of *Shigella flexneri* antigens by means of hemagglutination, bacterial agglutination, and complement-fixation tests yielded the following results.

1. The antigens of *Sh. flexneri* can be adsorbed on red blood cells of man, goat, rabbit, and guinea pig and are demonstrable by means of specific hemagglutination in the presence of homologous antibodies.

2. The supernates of cultures which were heated either at 100 C or at 52 C in neutral saline solution modify red blood cells. Suspensions treated at low pH fail to do so.

3. Data are presented regarding optimal conditions of preparation of antigens and of the hemagglutination test.

4. The hemagglutination test is not more sensitive for the detection of antigenic components of *Sh. flexneri* than the agglutination and agglutinin-absorption techniques, but is at least as effective as the complement-fixation test.

14. Park, B. Unpublished data.

CULTIVATION OF MOUSE-ADAPTED DENGUE VIRUS (TYPE 1) IN RHESUS MONKEY TISSUE CULTURE

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Although dengue virus has been successfully adapted to mice, attempts to cultivate it in tissue culture have so far yielded only negative or questionable results (Sabin, 1952; Hotta, 1952). The only reported exception is contained in a short discussion by Schlesinger (1950) of experiments in which dengue virus was propagated in 5-day-old chick embryo tissue.† In the present paper experiments are reported in which two strains of type 1 dengue virus were cultivated in tissue culture.

MATERIALS AND METHODS

Tissue culture.—Tissues used were from rhesus monkeys. Two different systems of tissue culture were investigated. One was testicular tissue embedded in chicken plasma clot and incubated in a roller apparatus (Smith and Evans, 1954). The second consisted of trypsin-treated kidney cells grown directly on a glass surface and incubated in the stationary state (Youngner, 1954). Either screw-capped or rubber-stoppered tubes, 16×150 mm, were used. Following incubation at 37 C for 4 to 5 days, tubes showing good cellular growth were selected for viral inoculation. Occasionally, they were stored at 10 or 20 C for a period not exceeding 1 week prior to inoculation with virus.

Medium.—For the initiation of cellular growth, or the storage of well-grown cultures, medium D of Youngner (mixture 199 with 2% inactivated horse serum) was used. The culture medium used after inoculation of virus was composed of 0.1% lactalbumin hydrolysate (Nutritional Biochemicals Corporation), 22.5% ox serum ultrafiltrate (Microbiological Associates), and 0.07% sodium bicarbonate in Hanks' balanced salt solution, with 200 units of penicillin and 200 μg of streptomycin per ml. It was necessary to continue cultures at least 3 weeks in order to observe the full effect of the virus. Therefore, a medium suitable for prolonged maintenance of cultures was required. Although comparative data were not collected on this point, the medium indicated above seemed to be distinctly better than certain other mediums. Neither 0.5% lactalbumin hydrolysate medium in Hanks' solution, medium D, nor mixture 199 alone were as satisfactory. The superiority of the first medium seemed more apparent with the kidney cell cultures than with testicular cultures. Phenol red was included in all mediums as a pH indicator.

Virus.—The viruses used were the mouse-adapted Mochizuki strain (Hotta, 1952) and the Hawaiian strain obtained from the American Type Culture Collection. Both had been passed more than 100 generations through mouse brain before the beginning of the present research. A homogenate was prepared containing approximately 10% glycerinated infected mouse brain in Ringer's solution and 20% inactivated normal horse serum. After centrifugation at 3000 r.p.m. for 15 minutes, the supernatant fluid was diluted appropriately and used as the initial virus suspension. Unless otherwise indicated, Ringer's solution, with 20% inactivated horse serum, was employed for dilution of viral materials.

Inoculation and incubation.—Inoculation of

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† After presenting a summary of this work at the meetings of the American Association of Immunologists in San Francisco in April, 1955, the authors had an opportunity to read a paper by Sabin (1955), in which it is briefly stated that dengue virus multiplied in monkey kidney tissue cultures without producing a cytopathogenic effect.

tissue cultures was carried out as follows in the initial cultivation and in the serial transmissions. Just prior to inoculation, the nutrient medium of the tissue cultures was discarded. Two-tenth ml of the appropriately diluted material, either mouse brain extract or tissue culture fluid, was put into each tube, and allowed to cover the cellular explants for about 10 minutes. Then 1.8 ml of the medium was added. The tubes were incubated at 35 C in a roller apparatus or in the stationary state. Either half- or whole-volume changes of medium were made at intervals of 2 to 7 days in order to keep the pH of the fluid phase within the range 7.2 to 7.6. The pH values were determined by comparison with a series of standard tubes containing phosphate buffer solutions and phenol red.

Assay of viral activity.—At suitable periods after the inoculation, portions of the culture fluid were taken from each tube and pooled with fluids from other tubes similarly inoculated. Serial 10-fold dilutions of the pools were then made, and 0.02 ml of each dilution was injected intracerebrally into 2- to 3-week-old white mice bred in this laboratory. All mice were checked daily for the tremor, flaccid paralysis and other signs characteristic of dengue infection. Mice which died within 3 days after the injection were not included in the results. The observation period was at least 3 weeks. LD_{50} values per 0.02 ml were calculated by the method of Reed and Muench (1938). The titrations were usually done immediately after harvesting the fluid. In a few instances the materials were sealed in pyrex glass tubes and stored in an iced thermos overnight or in a dry ice chest for periods not exceeding 4 months.

Morphological examination.—Following the viral inoculation, the cultures were checked daily for morphological changes visible under a microscope at magnifications of 32× and 100×. In the case of the trypsinized kidney cells, cultures in Porter flasks* and cover slip cultures in Leighton tubes* were also examined. The Porter flask cultures were observed in the living state with the ordinary microscope. The Leighton tube cover slip cultures were examined as follows: Cells growing on the surface of small cover slips (approximately 10×22 mm and 0.1 mm thickness) placed in the flat bottom of the tubes were infected with virus. At desired times the cover slips were removed and subjected to either staining or phase-contrast microscopy.† The staining procedures usually consisted of: fixation in Zenker-formol solution (Parker, 1950) for 30 minutes, washing in running water for 6 hours, and staining with May-Grunwald-Giemsa stain by a procedure slightly modified from that devised by Jacobson (Hanks et al, 1955). For phase-contrast microscopy the cover slip was placed on a drop of medium on a large cover slip (43×50 mm and 0.1 mm thickness). The cells were immersed in the thin layer of fluid between the two cover slips. This preparation was mounted on a metal slide with a central opening to accommodate the small cover slip. A second large cover slip was used to seal the opposite side of the hole in the metal slide. The metal slide and the large cover slips were sealed with paraffin. The technique is similar to that devised by Gey and described by Parker (1950).

Neutralization tests.—The antiserum was obtained from a rabbit immunized with Mochizuki strain of virus that had been passed only in mouse brain and never in tissue culture. Serum was collected from this rabbit prior to immunization to serve as a control nonimmune serum for the neutralization tests. The immunizing materials were supernatant fluids from the 10% infected mouse brain homogenates in Ringer's solution, centrifuged at 3000 r.p.m. for 15 minutes. The immunizing procedure consisted of the following injections: 3 times intravenously (0.5 ml to 0.7 ml, each), 5 times subcutaneously (1.0 ml to 2.0 ml, each), 4 times intraperitoneally (2.5 ml to 3.0 ml, each), and again 2 times intravenously (2.0 ml, each), at intervals of 5 to 10 days. The infective titers of suspensions for mice were presumed to be near 10^{-6} per 0.02 ml on the basis of occasional titrations. One week after the last injection, the rabbit was exsanguinated, and the serum was separated. The serum was inactivated by heating at 56 C for 30 minutes, and stored at −10 C until used. It was shown that the control serum had no neutralizing activity against the mouse-passaged type 1 dengue virus, and that approximately 5 LD_{50}'s of the mouse-passaged virus were neutralized completely by the immune serum diluted 320-fold.

In performing the neutralization test, virus at a given concentration was mixed in equal volumes with serum diluted 5- to 50-fold in lactalbumin medium. The mixture was kept at 37 C for 1 hour, and at 4 C for 1 more hour. Then 0.02 ml of the mixture was injected into mice intracerebrally or 0.2 ml was inoculated into tissue cultures with the subsequent addition of 1.8 ml of medium.

* May be purchased from Microbiological Associates.

† Assistance in microscopy given by Dr. Eichi Yamada, Department of Anatomy, University of Washington, is appreciated.

RESULTS

Control experiments

Dengue virus disappeared within a short period of time in culture medium without living cells. For instance, active virus was detected at 1 day after the inoculation, but not after 3, 7, 10 or 14 days, even when an inoculum with a mouse LD_{50} titer of $10^{-7.5}$ per 0.02 ml was used.

In every experiment 3 to 7 cultures with no virus were included. They were incubated and subjected to changes of medium exactly the same as infected cultures. They exhibited only slight degenerative changes after incubation which lasted for 3 to 10 weeks. It was repeatedly confirmed that homogenate of normal mouse brain diluted 1000-fold or more had no apparent effect on the culture cells.

Eighteen undiluted culture fluid specimens from various control experiments were injected intracerebrally into 5 or more 2-week-old mice. In no instance did death occur, except for a few mice which died presumably from the effect of trauma.

Ten serial blind passages were carried on through normal kidney tissue cultures, but no mouse-infective nor cytopathogenic agent was found in this experiment. A cytopathogenic agent isolated from a control kidney tissue culture will be discussed in a later section of this paper.

All specimens were inoculated onto bacteriological culture mediums and checked for bacterial or fungal growth. Contaminated tubes were infrequent and were not used to determine viral concentration or for passage.

TABLE 1.—*Mortality ratios of mice injected intracerebrally with culture fluid from dengue-infected rhesu testicular tissue cultures.*

Exp. no.	Seed virus	Dilution of culture fluid	Tissue culture incubation period in days: 1/12	1	3	5	7	8	10 Pooled	10 From a single tube	14 Pooled	14 From a single tube	17
I	Mochizuki 132nd mouse passage	10^{-6}	3/3	1/3	2/4	7/8	9/9	5/10	7/8	6/7	4/6	6/6	6/6*
		10^{-5}	3/3	0/3	1/4						3/5	2/6	3/6
	10^{-2} dilution	10^{-3}	1/3	0/3	0/4	1/8	2/9	0/9	2/8	4/7	1/5	0/6	1/5
	Mouse IC LD_{50}	10^{-3}	0/3	0/4	0/3								0/5
	Approx. $10^{-7.6}$	10^{-4}	0/3	0/3	0/3	0/8	0/8	0/8	0/7	0/7			0/5
		Calculated dilution of original inoculum in tissue culture fluid†	$10^{-4.0}$	$10^{-4.0}$	$10^{-3.0}$	$10^{-3.0}$	$10^{-3.0}$	$10^{-4.0}$	$10^{-4.0}$		$10^{-4.0}$		$10^{-4.0}$
			Tissue culture incubation period in days: 14		28		42	52	63		77	108	
II	Mochizuki 135th mouse passage	10^{-6}	5/6		5/5		6/6*	2/5	4/5*		4/6	0/6	
	10^{-2} dilution	10^{-1}	1/6		3/5		5/6	0/5	1/5		0/6	0/6	
	Mouse IC LD_{50}	10^{-2}	0/6		3/5			0/5					
	$10^{-6.5}$	10^{-3}						0/5					
		Calculated dilution of original inoculum in tissue culture fluid	$10^{-3.0}$		$10^{-3.3}$		$10^{-3.6}$	$10^{-3.0}$	$10^{-3.4}$		$10^{-3.7}$	$10^{-3.7}$	

Numerators indicate number of dead mice; denominators indicate number of injected mice. Fluid from replicate cultures were pooled except on the 10th and 14th day in experiment I, when tests were made of single tubes as well as from pools.

* Neutralization tests performed in mice identified the virus as dengue.

† The volume of medium removed and replaced varied; a 90% change was made on the 7th day, and a 50% change on the 14th day in experiment I. Changes of fluid in experiment II involved replacement of half volume or 90% of fluid at different times.

Dengue virus in rhesus testicular tissue culture

Two series of experiments were carried out, and the results obtained are summarized in table 1.

The data show that dengue virus remained active in rhesus testicular tissue cultures for a relatively long period. This observation appears to be significant when contrasted with the short survival time of a few days in control tubes lacking cells. In experiment II the successive changes of medium resulted in a dilution of the original inoculum beyond the original LD_{50} by the 42nd day. Virus was still present 3 weeks later in spite of further medium changes and resultant dilution of the original inoculum.

Passages were made at intervals of 8 to 14 days. All inoculated tubes of each subculture were incubated for 7 to 10 weeks. Tests for active virus in culture fluids were made every other week. Virus was detected only in the first 2 passages in the first experiment and the first 3 passages in the second experiment. It has not yet been determined whether this reflected the actual disappearance of virus or was the result of changes of the virus that reduced its virulence for mice.

Very few changes were noted in the appearance of fibroblastic cells in inoculated tissue cultures. In some cases certain degenerative alterations, such as cytoplasmic granulation and separation of cells, were seen, but they were hardly distinguishable from those found in the control tubes subjected to equally prolonged incubation.

Dengue virus in trypsin-treated rhesus kidney tissue culture

Results of a preliminary experiment with Mochizuki-strain virus of the 133rd mouse passage were strongly suggestive of an increase of virus in the fluid phase of rhesus kidney tissue cultures. Moreover, a distinct degeneration of the

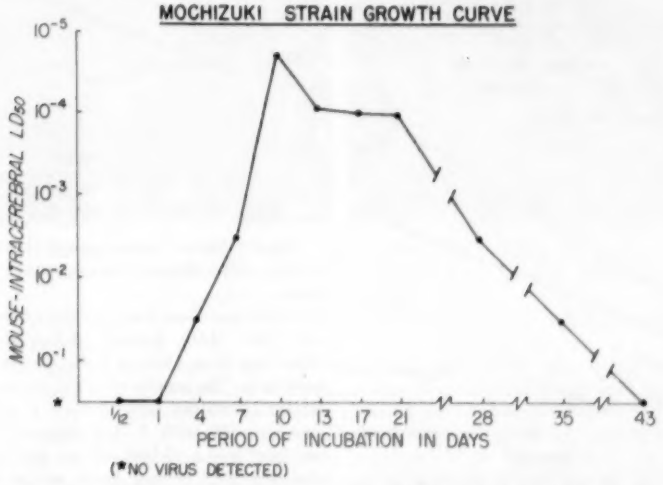


FIG. 1.—Growth curve of Mochizuki strain dengue virus in rhesus kidney tissue cultures.

Seven cultures were inoculated with 0.2 ml of a 10^{-4} dilution from a homogenate of infected mouse brain, 136th passage, with a mouse titer of $10^{-6.5}$ per 0.02 ml; 1.8 ml of culture medium was added to each tube. Vertical axis represents the titer of virus in pooled culture fluid removed at the times indicated.

cultured cells was noticed 1 to 3 weeks following the virus inoculation.

Growth curve.—Seven tubes were used for the infected group and 3 for the control. The initial virus inoculum was 0.2 ml of a 10^{-6} dilution from an infected mouse brain homogenate of Mochizuki-strain, 136th passage. Its intracerebral LD_{50} per 0.02 ml was $10^{-6.5}$; 1.8 ml of culture medium was added to each tube. At given periods following the inoculation, portions of the culture fluid were removed, pooled, and tested for mouse LD_{50}. The control tubes received a normal mouse brain homogenate and were treated the same as infected cultures. Figure 1 shows the results obtained. Increase of the virus was approximately 100,000-fold. The maximum titer of culture fluid was approximately one-hundredth of that of infected mouse brain. It is evident that the multiplication of dengue virus in rhesus kidney tissue culture is slow compared to other viruses such as poliomyelitis.

Serial transmission.—Results of serial transmission experiments with Mochizuki-strain virus in monkey kidney tissue cultures are illustrated in figure 2. This figure includes mouse titers in each passage, cumulative dilutions calculated on the basis of passages in successive tissue cultures, and cumulative dilutions based on both passages and changes of medium. After 10 passages during 120 days, 1 LD_{50} represented a dilution of 10^{-28} of the original inoculum.* Practically the same results were obtained with Hawaiian strain virus (see fig. 3).

By April 8, 1955, 31 passages were

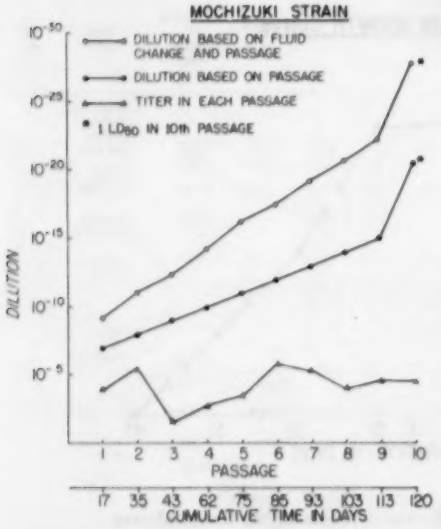


FIG. 2.—Serial transmission of Mochizuki strain dengue virus through rhesus kidney tissue cultures.

Initial inoculum was the same as that used in the growth curve experiment expressed in figure 1.

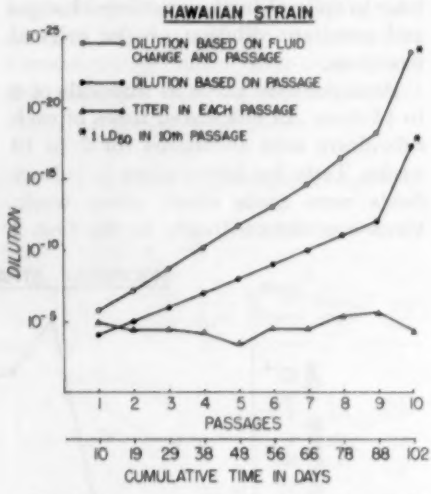
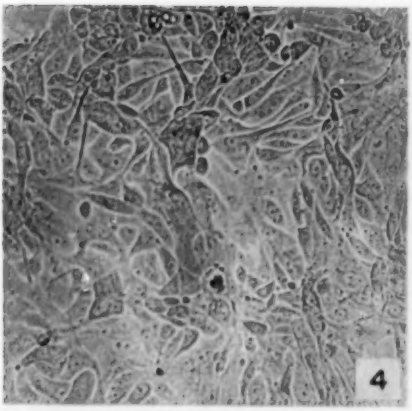
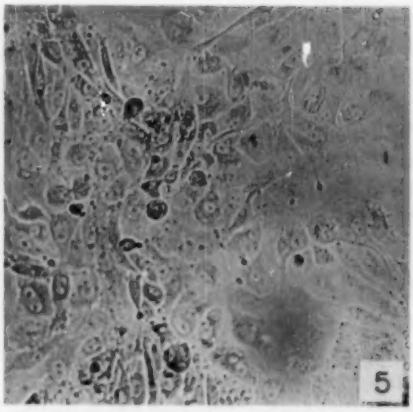


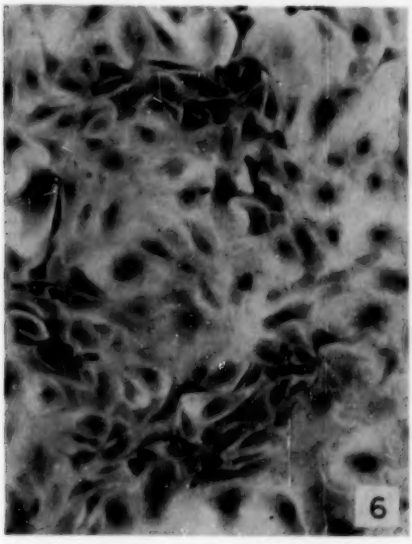
FIG. 3.—Serial transmission of Hawaiian strain dengue virus through rhesus kidney tissue cultures.

Initial inoculum was a mouse brain homogenate from 114th passage, delivered from the American Type Culture Collection in a lyophilized form. The powdered material was dissolved in this laboratory with medium E of Youngner (mixture 199 with 1.25 g sodium bicarbonate per liter) into a 10-fold volume, and diluted further with 20% normal horse serum in Ringer's solution. Two-tenth ml of a 10^{-3} dilution was inoculated, and 1.8 ml of culture medium was added in each tube.

* This degree of dilution was reached after 10 passages instead of 15 passages as stated previously (Hotta and Evans, 1955).







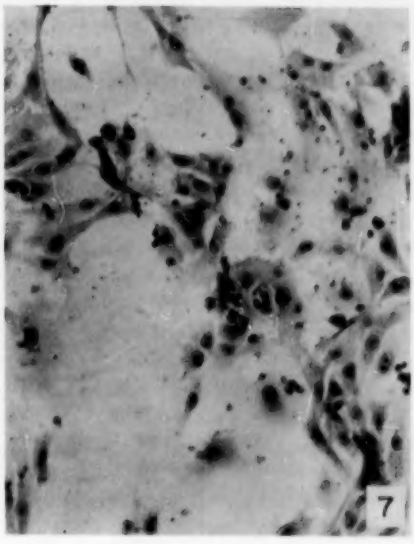


FIG. 4.—Normal rhesus kidney cells. Eighteen-day culture in a Porter flask. ×130.

FIG. 5.—Dengue-infected rhesus kidney cells in a Porter flask. Ten days after inoculation with Mochizuki strain virus. Twenty-third tissue culture passage. ×130.

FIG. 6.—Normal rhesus kidney cells. Twenty-day culture on a cover slip. Zenker-formol fixation, May-Grunwald-Giemsa stain. ×150.

FIG. 7.—Dengue-infected rhesus kidney cells on a cover slip. Twelve days after inoculation with Mochizuki strain virus. Fourth tissue culture passage. Zenker-formol fixation, May-Grunwald-Giemsa stain. ×130. Increase of the virus in this culture was confirmed as follows: 0.2 ml of undiluted fluid from the 3rd tissue culture passage was inoculated into this culture with the subsequent addition of 1.8 ml of lactalbumin medium. Mouse LD_{50} of the inoculum was $10^{-3.5}$ per 0.02 ml. The medium was changed every 2 to 4 days, and the resultant dilution of the original inoculum was $10^{2.2}$ fold by the time of photography. The fluid removed just before the photography showed a mouse LD_{50} titer of $10^{-5.25}$ per 0.02 ml.

carried out with Mochizuki-strain virus during 279 days, and 1 LD_{50} represented a 10^{-61} dilution of the original inoculum. The cultivated virus gave no indication of changed mouse infectivity up to that time.

Morphological changes.—The infected tissue culture cells exhibited degeneration visible under the microscope. The degeneration was first clearly detectable at 5 days to 1 week after the inoculation of virus. Affected cells were rounded and darkened. The general appearance under a magnification of 32× was that of black grains scattered on white cloth. The cells lost their firm attachment to the glass surface soon after they acquired the round dark appearance that identified them as undergoing degeneration. At 10 days it was common to see a culture in which the population of healthy cells was reduced by about half, and yet see very few degenerate cells. Presumably some cells had disintegrated and others had been washed off the glass surface in handling the tube. At 2 weeks only a relatively sparse population of cells usually remained. Some of these appeared normal; others exhibited long, slender strands or dense cytoplasmic masses. These changes were also regarded as signs of degeneration. The stained and phase-contrast preparations also showed these degenerative changes of infected cells. The normal-appearing cells that remained at that time subsequently underwent degeneration. Practically all cells disappeared after 3 to 4 weeks. No definite difference was noted in the degeneration pattern of the early and later passages, nor between Mochizuki and Hawaiian strains. During prolonged cultivation certain abnormal appearance developed in some cells of the uninoculated control tubes. A small number of cells became detached from the glass surface. However, the number was so small that an almost unbroken sheet of cells remained throughout the incubation. The control cultures never showed the distinct degeneration seen in the infected cultures. Figures 4 to 7 inclusive show typical examples of the control and infected rhesus kidney tissue culture cells.

Parallel titration in mice and tissue cultures.—The titers of tissue culture virus were compared in mice and in tissue cultures. Each tissue culture tube received 0.2 ml of inoculum; 0.02 ml was given each mouse. ID_{50} (50% infective dose) for tissue cultures was calculated on the basis of appearance of definite degeneration at 2 weeks after the inoculation. Examples of the results obtained are shown in table 2. Practically no difference was noted between the titers in mice and in tissue cultures.

FIG. 8.—Rhesus kidney cells grown on a cover slip and affected by a cytopathogenic agent. Ten days after inoculation. Second passage. Zenker-formol fixation, May-Grunwald-Giemsa stain. ×130.

TABLE 2.—*Parallel titrations of virus in mice and in kidney tissue cultures.*

Virus			Dilution							
Strain	Passage in tissue culture	Inoculated into*	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	LD_{50}	ID_{50}
Mochizuki	4	Mice	3/3†	3/3	3/3	1/3	0/3	0/3	$10^{-5.75}$	
		Tissue cultures	3/3†	3/3	3/3	2/3	0/3	0/3		$10^{-4.75}$
	30	Mice	6/6	6/6	5/5	2/5	1/5	0/5	$10^{-4.60}$	
		Tissue cultures	4/4	4/4	4/4	3/4	0/4	0/4		$10^{-4.50}$

* Inoculum =0.2 ml in tissue cultures with 1.8 ml of medium, and 0.02 ml in mice.
† Numerators indicate numbers of dead mice or tissue culture tubes showing definite degeneration. Denominators indicate numbers of mice or tissue culture tubes inoculated.

Identification of cultivated virus as dengue.—The cultivated virus was neutralized by the anti-dengue immune rabbit serum. The neutralization was clear in parallel tests with mice and tissue cultures. Table 3 shows examples of the results obtained.

A cytopathogenic agent recovered from an apparently normal kidney tissue culture

A cytopathogenic agent was originally encountered in a control uninoculated culture tube of rhesus kidney tissue 7 days after the beginning of incubation. The first sign noted was a small circular hole devoid of cells in a continuous sheet of cells. Surrounding this clear area was a zone of abnormal cells with numerous cytoplasmic vacuoles of various sizes, many larger than the nuclei. On the 8th day the discrete area had increased in size and become more distinct. Some of the surrounding cells appeared to have fused and formed a syncitial structure. Some of them lacked their original appearance, the cytoplasm having been replaced with huge vacuoles. The general view looked like a collection of bubbles. A subculture was made from the tube by transferring 0.2 ml of the undiluted culture fluid to new kidney tissue cultures, with subsequent addition of 1.8 ml medium. By April 8, 1955, 8 subcultures had been carried out in the same manner at intervals of 6 to 10 days. Similar morphological changes were found in each subculture. The clear areas became about 1 to 3 mm in diameter 7 to 10 days after the inoculation. At this time they were easily observed with the naked eye. In some cases there were 6 to 10 such areas in a single tube; in others they merged to form fewer or

TABLE 3.—*Neutralization tests to identify virus as type 1 dengue after passage in tissue culture.*

Virus				Immune serum* (anti-Mochizuki)		Control serum* (before immunization)	
Strain	Passage in tissue culture	Mouse IC LD_{50} per 0.02 ml	Dilution	Mouse	T.C.‡	Mouse	T.C.†
Mochizuki	6	$10^{-5.0}$	Undiluted	0/10‡	0/8‡	10/10	6/8§
	8	$10^{-4.0}$	Undiluted	0/5	0/8	5/5	8/8
	28	$10^{-6.0}$	10^{-3}	0/7	0/4	6/7	5/5
Hawaiian	2	$10^{-4.0}$	Undiluted	0/5	0/6	5/5	7/7
	8	$10^{-5.50}$	10^{-3}	0/7	0/5	7/7	5/5

* Diluted 5 times with 0.1% lactalbumin hydrolysate medium.
† The culture fluid used for changing medium included 1% of immune or control serum.
‡ Numerators indicate numbers of dead mice or tissue culture tubes showing definite degeneration. Denominators indicate numbers of mice or tissue culture tubes inoculated.
§ Two tubes showed nonspecific degeneration.

even a single large hole in the sheet of cells. Cells not immediately adjacent to such areas were apparently normal at that time. However, the whole cellular explants underwent degeneration after 2 to 3 weeks. Figure 8 shows an example of the typical findings.

Neither bacterial nor fungal growth were found in bacterial culture mediums inoculated with the culture fluids from these tubes.

The undiluted culture fluid from 7th subculture was injected intracerebrally into 14 2-week-old white mice. Neither death nor specific symptoms were noticed during a 4-week observation period.

The anti-Mochizuki immune rabbit serum did not affect the cytopathogenicity of the unknown agent for tissue cultures. It was apparent, therefore, that no antigenic relationship between this cytopathogenic agent and dengue virus was detected.

DISCUSSION

Whether the rhesus testicular tissue cultures supported growth of dengue virus remains uncertain. Evidence of multiplication of virus in this system was equivocal. No distinct cytopathogenic effect was noticed. It is clear, however, that the virus survived in testicular cultures at least 11 weeks, whereas it disappeared completely in a few days from tubes containing tissue culture mediums without cells.

In contrast to the results with testicular tissue, it was evident that rhesus kidney tissue cultures supported definite multiplication of dengue virus. Identical results were obtained with 2 strains of virus from different sources. A marked feature of the growth pattern associated with dengue virus was its slowness. Degenerative changes were seen in the infected cells. Although some uninoculated control cultures showed a relatively limited change after prolonged incubation, they were far less extensive than those observed in the infected cultures and never equaled the widespread destruction of cells seen invariably in cultures exposed to dengue virus. Moreover, specific immune serum prevented growth of dengue virus and degeneration of cells. It was unequivocally established, therefore, that dengue virus produced a cytopathogenic effect on the trypsin-treated rhesus kidney cells. It should be noted from the growth curve experiment that maximal titers of virus were found in the tissue culture fluid at about 7 to 10 days when degeneration was beginning to appear. The possibility that much virus is released prior to development of obvious degenerative changes must be considered. After 10 days, apparently release of virus was offset by inactivation of virus in the fluid medium.

The cytopathogenic agent recovered from apparently normal kidney tissue cultures fortunately was not a source of confusion. It was clearly distinguished from dengue virus by mouse-infectivity tests, neutralization tests, and morphological examination of the cells. Probably this agent is similar, if not identical, to that described by Rustigian et al (1955). The possibility that a mixed infection with dengue virus and this agent occurred in a single culture cannot be excluded with certainty. It is curious that this agent was encountered only once in the dengue-infected cultures, but more often in the uninoculated control cultures. Culture fluid from the dengue-inoculated tube showing signs of concomitant infection with this agent and dengue virus was transferred to new cultures, and serial transmissions were performed. From the 2nd to 4th subcultures the degeneration pattern showed both the large vacuolated multinuclear cells induced by the un-

named agent and the small round dark cells associated with dengue infection. After the 5th subculture the affected cells showed only changes similar to the original dengue infection. Mouse-infectivity tests showed that dengue virus was retained throughout these subcultures. It appeared that dengue virus might have excluded the other agent.

Finally, it should be kept in mind that both strains of dengue virus used in the present study were mouse-adapted. In view of the difficulty of isolating dengue virus from natural sources by inoculation of mice, trials to isolate it with tissue cultures should be undertaken.

SUMMARY

The in vitro cultivation of mouse-adapted dengue virus was studied with rhesus monkey testicular and kidney tissue cultures.

In testicular tissue cultures embedded in chicken plasma clot and incubated in a roller apparatus at 35 C, dengue virus of Mochizuki-strain persisted in the active state at least 11 weeks. It was not determined, however, whether or not the virus multiplied.

In the trypsin-treated kidney tissue cultures grown directly on glass surface and incubated in the stationary state at 35 C, both the Mochizuki and Hawaiian strains of type 1 virus multiplied. The multiplication was relatively slow, at least under the conditions adopted. The Mochizuki strain was maintained through 31 passages and the Hawaiian strain through more than 10 passages. Infectivity for mice was shown with tissue culture fluids representing $10^{-6.1}$ dilution of the Mochizuki strain inoculum and $10^{-2.6}$ dilution of the Hawaiian strain inoculum.

The cultivated virus retained its original mouse infectivity, and was neutralized by the specific immune rabbit serum. The infected cells exhibited characteristic degeneration which also was suppressed by specific immune serum.

A cytopathogenic agent was recovered from an apparently normal kidney tissue culture. This agent was distinguished from dengue virus biologically and immunologically.

REFERENCES

Hanks, J. H. et al. 1955, An Introduction to Cell and Tissue Culture, Minneapolis, Burgess.

Hotta, S. 1952, Experimental studies on dengue. I. Isolation, identification and modification of the virus. J. Infect. Dis. **90**: 1–9.

Hotta, S. and Evans, C. A. 1955, Cultivation of mouse-adapted dengue virus in tissue culture, Fed. Proc. **14**: 466.

Parker, R. C. 1950, Methods of Tissue Culture, ed. 2, New York, Hoeber.

Reed, L. J. and Muench, H. 1938, A simple method of estimating fifty per cent endpoints. Am. J. Hyg. **27**: 493–497.

Rustigian, R., Johnston, P. and Reihart, H. 1955, Infection of monkey kidney tissue cultures with virus-like agents. Proc. Soc. Exper. Biol. & Med. **88**: 8–16.

Sabin, A. B. 1952, Research on dengue during World War II. Am. J. Trop. Med. & Hyg. **1**: 30–50.

———. 1955, Recent advances in our knowledge of dengue and sandfly fever. Am. J. Trop. Med. & Hyg. **4**: 198–207.

Schlesinger, R. W. 1950, Propagation in chick embryos of the Hawaiian strain of dengue virus. I. Sustained serial passage in eggs after one hundred and one intracerebral passages in mice. Am. J. Hyg. **51**: 248–254.

Smith, W. M. and Evans, C. A. 1954, Growth of neurotropic viruses in extraneural tissues. VII. Cultivation of MM and western equine encephalitis viruses in cultures of rhesus testicular tissue. J. Immunol. **72**: 353–359.

Youngner, J. S. 1954, Monolayer tissue cultures. I. Preparation and standardization of suspensions of trypsin-dispersed monkey kidney cells. Proc. Soc. Exper. Biol. & Med. **85**: 202–205.

TRANSMISSION STUDIES WITH ENCEPHALOMYOCARDITIS VIRUS

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Some of the viruses of the encephalomyocarditis (EMC) group are known to be pathogenic to man.[1,2] Isolations of the virus have been made from the rhesus monkey,[3] chimpanzee,[4] mandrill baboon,[5] cotton rat,[6] hamster,[7] mongoose, and wild-caught mosquitoes of the genus *Mansonia*.[8] Certain epidemiological observations have tended to incriminate rats as the reservoir of the virus, and antibodies to this virus group have been found in the serums of wild rats collected in Africa and North America, while serums from other animal species collected in the same areas have been negative for antibody.[8,9] While presence of virus in wild-caught mosquitoes might indicate an arthropod-borne type of transmission, the presence of virus in the mosquito could be purely adventitious. The limitation of the presence of naturally occurring antibody to the serums of rats, to the exclusion of other small animals, is not in conformity with most other mosquito-borne virus diseases.

The work to be described was undertaken to test several possible modes of transmission of encephalomyocarditis virus among rodents.

MATERIALS AND METHODS

Virus.—The strain of EMC virus used was that isolated from a mandrill baboon from a zoological park in Florida.[8] All tests were made with third and fourth mouse brain passage material.

Titration of virus.—Titrations of virus-containing materials were made intracerebrally in 3-week-old mice. Four or 5 mice were used on each dilution. The LD_{50} end points were calculated by the method of Reed and Muench.[10]

Animals.—Mice used were CFW or CF-1 strains. Guinea pigs were from the stock raised at this laboratory. Cotton rats were obtained from commercial sources.

Mosquitoes.—The *Aedes aegypti* used in these experiments were reared in the laboratory from a strain originally obtained from Technical Development Laboratories, Technology Branch, Communicable Disease Center, Savannah, Georgia. The *Mansonia perturbans* (Walker) were caught as adults near Ponchatoula, Louisiana. All mosquito incubations were at 80 F and relative humidity of about 75%.

EXPERIMENTAL

Viremia studies.—Preliminary to transmission attempts with mosquitoes, the following tests were run to determine the presence, duration, and amount of virus in the blood of several species of rodents to aid in the selection of an appropriate host animal.

Four guinea pigs were inoculated subcutaneously with 6300 mouse intracere-

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1. Smadel, J. E. and Warren, J. 1947, J. Clin. Investigation, **26**: 1197.
2. Dick, G. W. A., Haddow, A. J., Best, A. M. and Smithburn, K. C. 1948, Lancet, **2**: 286–289.
3. Dick, G. W. A., Smithburn, K. C. and Haddow, A. J. 1948, British J. Exper. Path. **29**: 547–558.
4. Helwig, F. C. and Schmidt, E. C. H. 1945, Science, **102**: 31–33.
5. Kissling, R. E. and Vanella J. M. To be published.
6. Jungeblut, C. W. and Sanders, M. 1940, J. Exper. Med. **72**: 407–436.
7. Jungeblut, C. W. and Dalldorf, G. 1943, Am. J. Pub. Health **33**: 169–172.
8. Rivers, T. M. 1952, Viral and Rickettsial Infections of Man, ed. 2, Philadelphia, J. B. Lippencott Co., p. 678.
9. Warren, J., Russ, S. B. and Jeffries, H. 1949, Proc. Soc. Exper. Biol. & Med. **71**: 376–387.
10. Reed, L. J. and Muench, H. 1938, Am. J. Hyg. **27**: 493–497.

bral LD_{50}'s and blood was collected from them at regular intervals from the 6th to 192nd hour after inoculation. The bloods thus collected were qualitatively tested for presence of virus by inoculation of a 10^{-1} dilution intracerebrally into mice. One of the guinea pigs failed to show a febrile response or to circulate virus, and the other three showed slight febrile responses and circulated virus for 48 to 120 hours (table 1). There was

TABLE 1. *Presence of virus in the blood of guinea pigs inoculated subcutaneously with EMC virus.*

Guinea pig no.	Hours after inoculation								
	6	24	48	72	96	120	144	168	192
1	−	−	−	±	+	+	−	−	−
2	−	−	+	+	−	+	+	+	−
3	−	−	+	+	+	+	−	−	−
4	−	−	−	−	−	−	−	−	−

seldom sufficient virus to kill all the mice inoculated with the 10^{-1} dilution of blood. In view of their relative insusceptibility, no further work was done with guinea pigs.

Ten 3-week-old mice were inoculated subcutaneously with 6300 mouse intracerebral LD_{50}'s. Two of the mice were bled (and sacrificed) at 6 hours, and 2 others each day thereafter through 72 hours. Three mice were bled at the 72-hour period, since of the 4 mice left at this time, 1 was dead and the other 3 were prostrate. Virus was found in the 10^{-1} dilution of blood of all the mice bled at the various time intervals.

Ten other mice were inoculated and bled in a similar manner, but the blood samples were titrated intracerebrally in mice. The highest blood virus titer was $10^{6.5}$, found in one mouse at 48 hours after inoculation (table 2).

Several cotton rats were inoculated subcutaneously with a 10^{-3} dilution of EMC-infected mouse brain containing approximately 6000 mouse intracerebral LD_{50}'s of virus. Blood samples were

TABLE 2.—*Virus titers in blood of mice inoculated subcutaneously with EMC virus (baboon strain).*

Mouse no.	Hours after inoculation				
	2	24	48	72	96
1	0				
2	0				
3		3.7*			
4		3.0			
5			5.7		
6			6.5		
7				5.5	
8				5.0	
9					4.3
10					Dead

* Log LD_{50}.

taken from two different animals of this group each day after inoculation and titered for virus content. The amount of virus was similar to that found in the blood of infected mice. The cotton rats, however, succumbed to infection sooner than did the mice (table 3).

The viremia levels produced in mice by the recently isolated baboon strain of EMC virus were compared with those produced by the original strain which had been isolated in 1948 from a chimpanzee[4] but had received numerous intracerebral mouse passages. The mice were inoculated with a higher dilution of virus (10^{-6}) to insure survival through 72 hours. It can be seen in table 4 that the level of circulating virus produced by this mouse adapted strain was not significantly different from those levels resulting from infection with the recently isolated strain (table 2).

TABLE 3.—*Virus titer in blood of cotton rats inoculated subcutaneously with EMC virus (baboon strain).*

Cotton rat no.	Hours after inoculation			
	0	24	48	72
1	0			
2	0			
3		4.0*		
4		4.8		
5			5.8	
6			5.5	
7				6.8
8				Dead
9				Dead
10				Dead

* Log LD_{50}.

TABLE 4.—*Viremia levels produced in mice after subcutaneous inoculation of a mouse-adapted strain of EMC virus.*

Mouse no.	Hours after inoculation				
	2	24	48	72	96
1	0				
2	0				
3		5.5*			
4		0			
5			5.5		
6			5.2		
7				5.3	
8				4.5	
9					5.2

* Log LD_{50}.

Mice were considered to be the animal of choice for the mosquito transmission experiments because of the blood virus levels produced, their availability, and ease of handling.

Mosquito transmission attempts with A. aegypti.—One hundred and eighty-four *A. aegypti* mosquitoes were allowed to take blood meals from mice circulating virus in titers of $10^{4.7}$ to $10^{5.5}$ (table 5). Sample lots of these mosquitoes were tested for virus by intracerebral mouse inoculation after 6 and 15 days of incubation, with negative results. Attempts to transmit infection by bites of 30 to 70 mosquitoes were made after extrinsic incubation periods of 10, 21, and 27 days. No transmission was effected; and when these mosquitoes were subsequently ground and inoculated into mice, only one pool of 10 mosquitoes in the 27-day group contained a trace of virus.

Mosquito transmission attempts using M. perturbans.—Fifty-one *M. perturbans* were allowed to engorge upon mice circulating virus in titers of $10^{4.8}$ to $10^{5.6}$. The mortality rate in the mosquitoes subsequent to this feeding was rather high, probably because they were getting old. They had been captured as adults and therefore their ages were unknown at the time of the tests. Twelve mosquitoes dying between the 8th and 11th days of incubation were tested for virus and found negative.

TABLE 5.—*Viremia levels in mice at time of mosquito feeding* (A. aegypti).

Mouse no.	Log-virus titer of blood	Number of mosquitoes feeding
1	5.7	12
2	6.5	9
3	5.8	10
4	6.5	26
5	5.5	22
6	4.7	27
7	1.7	28*
8	5.7	27
9	5.5	28
10	5.5	23

* These mosquitoes discarded from experiment.

After a 9-day extrinsic incubation period, 11 mosquitoes from a group of 17 refed singly upon normal mice. No transmissions occurred and no virus could be demonstrated in suspensions of any of the 17 mosquitoes.

Excretion of virus by mice.—Preliminary toattempting contact transmission, infected mice were tested for the presence of virus in the urine and feces. Five mice were inoculated subcutaneously with 6000 intracerebral LD_{50}'s of virus. Urine and feces were collected daily thereafter and tested for the presence of virus. The mice usually urinated and defecated promptly upon being picked up. The urine was collected in a petri dish containing a small amount of saline and the feces were collected on a swab and transferred to a tube. Antibiotics were added to control bacterial contaminants. Four of the mice survived through 96 hours, while one died of infection after 72 hours. Virus was present in both the urine and feces of all 5 mice on each day except for a negative fecal specimen from one mouse collected 72 hours after inoculation. The urine specimen from one mouse and the fecal specimen from another could not be obtained at 72 hours.

Mouse contact transmission studies.—Ten 3-week-old mice were inoculated intranasally with 0.03 ml each of a 10^{-2} dilution of virus, representing approximately 60,000 intracerebral LD_{50}'s.

Five each were placed in a box containing 5 normal mice. The feed was supplied in an overhead hopper and the water in the usual type bottle. All of the inoculated mice died of infection between the 3rd and 5th days. The contact mice survived through 24 days without showing signs of disease, and at that time they were challenged intracerebrally with 60 LD_{50}'s of virus. All succumbed to challenge.

In a second test, ten 3-week-old mice were inoculated subcutaneously with 6000 intracerebral LD_{50}'s of virus. Five each were placed in a box in contact with 5 normal mice of the same age. The feed was given on the bottom of the box so that contamination with urine and feces was possible. In one box all of the inoculated mice died of infection between the 4 and 6th days after inoculation. The contact mice survived through the 19th day and at that time were challenged with a small dose of virus. Four of the 5 contacts died following challenge. Seven of 9 non-contact controls died from the same challenge.

In the other box, 4 of the 5 inoculated mice died of infection between the 4th and 6th days after inoculation. The 5th lived through the 19th day, at which time it survived intracerebral challenge with virus. One of the inoculated mice which died on the 5th day was partially eaten by its cagemates. Subsequently, 3 of the contact mice died with symptoms characteristic of EMC infection. One of the 2 surviving contacts withstood an intracerebral challenge with virus.

Cotton rat contact transmission studies.—Five cotton rats were inoculated intranasally with 0.03 ml of a 10^{-3} dilution containing 6000 mouse intracerebral LD_{50}'s of virus. Each rat was then placed in a cage with a normal cotton rat. The inoculated rats died between the 2nd and 4th day after inoculation. No cannibalism occurred. Three of the 5 contact rats developed EMC infection and died on the 6th, 9th, and 11th days after being placed in contact with the inoculated rats.

Transmission to mice by ingestion of virus.—In view of the transmission of EMC virus in a box of mice in which cannibalism occurred, the following experiment was performed:

Each of 9 three-week-old mice was fed one-half of an EMC-infected mouse brain. The alternate brain halves were pooled and titered intracerebrally in mice with a resultant LD_{50} at $10^{-7.5}$. Seven of the 9 mice died of EMC infection between the 3rd and 8th day after eating the infected material. The two surviving mice succumbed to a challenge of EMC virus given 22 days later.

DISCUSSION

Two mosquito species, *A. aegypti* and *M. perturbans*, failed to transmit EMC virus after feeding on infected mice. However, the attempt using *Mansonia* may be considered incomplete since a high mortality in the mosquitoes restricted the extrinsic incubation period to only 9 days. On the other hand, the fact that none of the *M. perturbans* retained the virus would tend to substantiate their inability to transmit even had a longer extrinsic incubation period been allowed.

The present limited experiments do not rule out mosquitoes as vectors of EMC virus. If arthropods are involved, however, it would be more logical to suspect a more host-specific one than mosquitoes, in view of the results of reported serological surveys in both the United States and Africa. Furthermore, Gillett and Dick have found that *Mansonia fuscopennatus*, *M. uniformis*, and *M. africanus* are incapable of transmitting African EMC (Mengo) virus. In fact, these mosquitoes retained virus in

their bodies for no more than 8 days following an infected meal.[11]

The ability of cotton rats to transmit the virus by contact tends to place transmission by this means under greater suspicion as a "natural" method than transmission by arthropod vectors. The cannibalistic tendencies of many rodents and the experimental proof of infection of mice through ingestion of infected brain material indicate a supplementary means of transmission.

Since EMC virus is excreted in the urine and feces of mice, it would seem advisable to investigate food contamination through these sources in the event of future outbreaks in humans.

SUMMARY

Encephalomyocarditis virus infections were transmitted in cotton rats by contact. Albino mice became infected through ingestion of virus-containing material but not by direct contact although virus was excreted in the urine and feces of infected mice.

Transmission attempts using *Aedes aegypti* and *Mansonia perturbans* failed.

11. Dick, G. W. A. 1953, Tr. Roy. Soc. Trop. Med. & Hyg. **47**: 13–48.

STUDIES ON *PASTEURELLA PESTIS* IN VARIOUS FLEA SPECIES

I. INFECTION OF FLEA SPECIES WITH AVIRULENT STRAINS OF *PAST. PESTIS*

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Bacot and Martin (1914), in a published account of their investigations concerning the mechanism of transmission of *Pasteurella pestis* by infective fleas, were of the opinion that the organisms are introduced into a susceptible animal through a process of regurgitation due to a temporary obstruction at the entrance to the stomach. The multiplication of the plague bacillus in the gut of the flea, especially in the esophagus and proventriculus, caused occulsion of the lumen of the proventriculus, and the subsequent act of feeding by a flea caused regurgitation of the organisms into the host. Bacot (1915) points out that the initial stage of the blockage is due to the lodgment and growth of the bacilli among the proventricular spine-like epithelial cells. The classical work of Bacot and Martin was conducted with two species of fleas, namely *Xenopsylla cheopis* and *Nosopsyllus fasciatus*. Since then, Eskey (1938), Eskey and Haas (1940), Douglas and Wheeler (1943), Burroughs (1947), and Holdenried (1952) have shown experimentally that flea species other than *X. cheopis* and *N. fasciatus* are efficient plague vectors.

Due to the apparent paucity of literature relative to investigating the phenomenon of gastrointestinal blockage in flea species with avirulent plague bacilli, investigations concerning the possible development of gastrointestinal blockage in fleas by avirulent strains of *Past. pestis* were undertaken by the Department of Entomology in cooperation with the Department of Bacteriology.

While the studies were in progress, Quan, Kartman and McManus (1954) observed blockage of the digestive tract of the flea species *X. cheopis* in experiments in which fleas received their infectious blood meal by feeding through an animal skin membrane in contact with blood containing avirulent plague organisms. Blockage, characteristic of the blocks formed by virulent strains of *Past. pestis*, were observed as early as the second day following the infectious meal.

Our studies, though similar in nature and differing only in the mode of infecting fleas, were based independently on the same premise as that of Quan et al (1954), and form the basis for the observations reported here.

MATERIALS AND METHODS

Avirulent plague strains EV51, TRU and A1122, and the following species of laboratory colonized flea species were used in these studies: *Monopsyllus anisus* (Tokyo, Japan), *M. anisus* (Kobe, Japan) and *X. cheopis* (San Francisco, California). White mice, locally procured, were used as the experimental animals. Specially designed cages (fig. 1 and 2) were used to individually confine white mice in flea infection and transmission procedures.

The concentration of organisms in the inocula varied from 2×10^4 to as high as 545×10^6 for inoculation of animals. Route of inoculation of ex-

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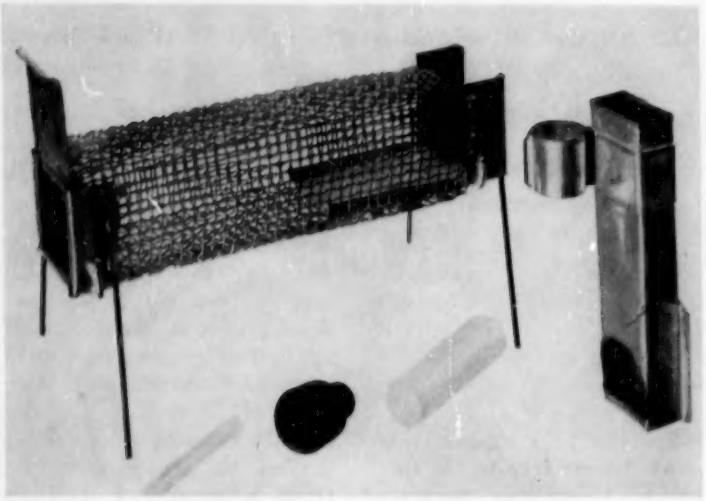

FIG. 1.—Dismantled mouse confinement cage.

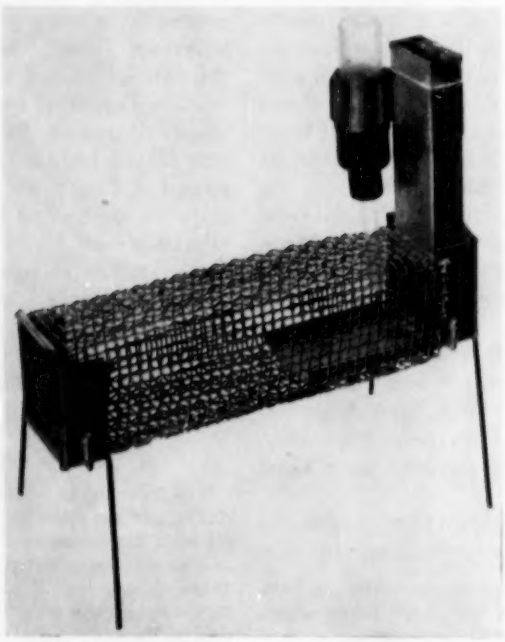

FIG. 2.—Assembled mouse confinement cage.

perimental animals also varied, but the majority of animals received the inoculum via the tail tissues. Some animals, however, received the inoculum intraperitoneally, subcutaneously or by formation of an intradermal bleb on the belly of the animal.

The laboratory-reared fleas used in the experiments were starved for a period of 24 hours prior

to feeding. In the majority of the experiments 50 to 100 fleas were permitted to feed upon infected animals. Only those fleas observed to have obtained a blood meal were used. Fleas suspected of having become infected were examined microscopically for detection of any developing mass in the digestive tract. Suspiciously blocked fleas were segregated and divided into two lots. One lot was fed on normal mice and examined daily for possible proventricular blocking and subsequent transmission. Three to 5 fleas of the second lot were selected at daily intervals, dissected and the proventriculus and ventriculus of each removed. These organs from dissected fleas, as well as from any suspiciously blocked fleas of the first lot, were triturated separately in a Ten Broeck tissue grinder in a small amount of 1% peptone water and portions of the material cultured in tryptose phosphate broth and streaked on blood agar plates. A second portion was inoculated into normal mice via the subcutaneous route. Cultures and slide preparations were made of spleen and heart material removed from any mice which succumbed during the course of the study. The remainder of these organs were ground, emulsified and inoculated into susceptible animals. Mice surviving were held for a period of at least 14 days, then sacrificed and examined. Suspicious autopsy material was passed to other susceptible mice. Slide preparations were stained with Wayson's stain and examined microscopically. Mice on which fleas had fed were observed for developing symptoms of plague. Bacteriological identification tests were performed by the Department of Bacteriology.

RESULTS

Transmission attempts.—Fleas used in these experiments received their infectious blood meal from mice previously inoculated via the subcutaneous tissue of the tail with 0.5 ml broth emulsion of the avirulent plague strains cultured at 30 C. This route of inoculation was preferred because the inoculated animals survived for 3 to 5 days. Fleas were placed on infected mice 24 hours after inoculation via the tail tissue since qualitative determination of bacteremia in mice inoculated in this manner, with strain EV51, revealed organisms present in the circulating blood and also in the spleen at that time. Prior to this period organisms were not found in the blood nor in the spleen. After 48 hours isolates of *Past. pestis* were obtained from the spleen but not from the heart blood. The infected host mice were placed in confinement cages within glass battery jars and 50 to 100 fleas per mouse permitted to feed until each animal exhibited severe illness or until it died.

Fleas removed from the inoculated animals were placed upon new susceptible animals and permitted to feed. Mice developing signs and symptoms of plague were sacrificed, or upon death autopsied, grossly examined and emulsions of heart and spleen passed to normal animals. A second normal animal was placed with each lot of fleas and the procedure repeated as long as any fleas remained alive.

Transmission attempts were negative in which the various flea species in combination with the different plague strains were used. Negative results were also obtained in experiments in which fleas were afforded the opportunity to feed on mice treated by X-ray irradiation or by intramuscular injection of cortisone acetate or fraction IA (*Past. pestis*) in an attempt to depress the reticulo-endothelial system activity prior to inoculation with an avirulent plague strain.

Blockage of digestive tract of fleas.—The degree of bacteremia determined by tail vein smear in inoculated mice was usually found to be low. The count never averaged more than two organisms per high power microscopic field. Use of reticulo-endothelial depressants, such as 96 roentgen units of X ray per mouse, 0.5 mg fraction IA per mouse, or 1.5 mg of cortisone acetate per mouse, administered prior to inoculation of animals failed to produce the desired high bacteremic count in these animals. Despite the low degree of bacteremia in inoculated animals, isolates of *Past. pestis* were nevertheless obtained from the proventriculus of *X. cheopis* (Cali-

fornia) and from the ventriculus of *M. anisus* (Tokyo).

In one experiment a white mouse was inoculated via the subcutaneous tissue of the tail with a suspension of A1122, the inoculum containing 243 × 10^6 organisms. After feeding on the inoculated mouse for 48 hours, two *X. cheopis* with dark proventriculi and empty constricted ventriculi were examined under the phase microscope. Under microscopic examination the proventricular teeth were not discernible, apparently obscured by the developing proventricular mass. When proventriculi of the two fleas were dissected out and microcultured on a slide, isolates of *Past. pestis* were obtained in both instances.

With *M. anisus*, in 3 of 13 experiments performed, although no proventricular blocks were noted to develop, dark, free-floating masses were observed in the ventriculi of the fleas in nine instances. Fleas received their infectious blood meals from white mice previously inoculated via the subcutaneous tissue of the tail with an emulsion of a 48-hour culture of the strain EV51. The isolates of *Past. pestis* were obtained from fleas which had fed for 3 days on a mouse inoculated with 447 × 10^6 organisms. Three days after their last blood meal, six fleas were examined microscopically and then dissected for culture. Two isolates were obtained from the dissected fleas.

DISCUSSION

Inducement of a desired high degree of bacteremia in animals, inoculated via different routes with the various avirulent plague strains used in the studies, was unattainable irrespective of the concentration of the inoculum with or without the aid of reticulo-endothelial depressants such as X ray irradiations, fraction IA or cortisone acetate. The series of experiments conducted by us have resulted in low bacteremic count in animals inoculated with avirulent plague organisms. Therefore, the method employed by us in attempts to mass infect fleas is contraindicated and a more feasible method of mass infection of fleas with avirulent plague organisms appears to be that employed by Quan et al.

SUMMARY

Low bacteremic count in animals inoculated with avirulent plague organisms resulted in failure to mass-infect fleas. Nevertheless, two *X. cheopis* revealed proventricular blockage from which isolates of *Past. pestis* were obtained, while two isolates of *Past. pestis* were obtained from six *M. anisus* which developed dark, free-floating masses in the ventriculus. All transmission attempts were negative.

REFERENCES

Bacot, A. W. and Martin, C. J. 1914, Observations on the mechanism of the transmission of plague by fleas. J. Hyg. Plague Suppl. 3: **13**: 423–439.

Bacot, A. W. 1915, Further notes on the mechanism of the transmission of plague by fleas. J. Hyg. Plague Suppl. 4, **14**: 774–776.

Eskey, C. R. 1938. Recent developments in our knowledge of plague transmission. Pub. Health Rep. **53**: 49–57.

Eskey, C. R. and Haas, V. A. 1940, Plague in the western part of the United States, Pub. Health Bull. **254**: 83 pp.

Douglas, J. R. and Wheeler, C. M. 1943, Sylvatic plague studies. II. The fate of *Pasteurella pestis* in the flea, J. Infect. Dis. **72**: 18–30.

Burroughs, A. L. 1947, Sylvatic plague studies. The vector efficiency of nine species of fleas compared with *Xenopsylla cheopis*. J. Hyg. **45**: 371–396.

Holdenried, R. 1952. Sylvatic plague studies. VII. Plague transmission potentials of the fleas *Diamanus montanus* and *Polygenis gwyni* compared with *Xenopsylla cheopis*. J. Infect. Dis. **90**: 131–140.

Quan, S. F., Kartman, L. and McManus, A. G. 1954, Studies on *Pasteurella pestis* in fleas. II. Experimental blocking of *Xenopsylla cheopis* with an avirulent strain of *Past. pestis*. Science, **120**: 1101–1102.

STUDIES ON *PASTEURELLA PESTIS* IN VARIOUS FLEA SPECIES

II. SIMPLIFIED METHOD FOR THE EXPERIMENTAL INFECTION OF FLEAS

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It became evident early in the studies that avirulent plague organisms never produce a high degree of bacteremia in animals inoculated via different routes irrespective of the concentration of the inoculum. Use of reticulo-endothelial depressants, such as X-ray irradiation, fraction IA (*Pasteurella pestis*) or cortisone acetate administered prior to inoculation of animals failed to produce the desired high bacteremic count in these animals. Cortisone acetate, however, may have depressed the reticulo-endothelial system to the extent that isolation of plague organisms from the various organs was facilitated and haemorrhagic infiltration of the subcutaneous tissue of the mouse was induced more rapidly following inoculation with avirulent plague organisms. Since attempts to infect flea strains by allowing the fleas to feed on an animal inoculated with an avirulent plague organism were unsuccessful due to the low bacteremic count in the infected animal, a simple method of infecting fleas in vitro was devised. While these studies concerning artificial methods of infecting fleas were in progress, Kartman (1954) reported upon an artificial method for the feeding of fleas. This method was based upon the principle of transferring controlled thermal gradients from a waterbath to a metal dish containing blood upon which fleas were allowed to feed through an animal skin membrane in direct contact with the blood.

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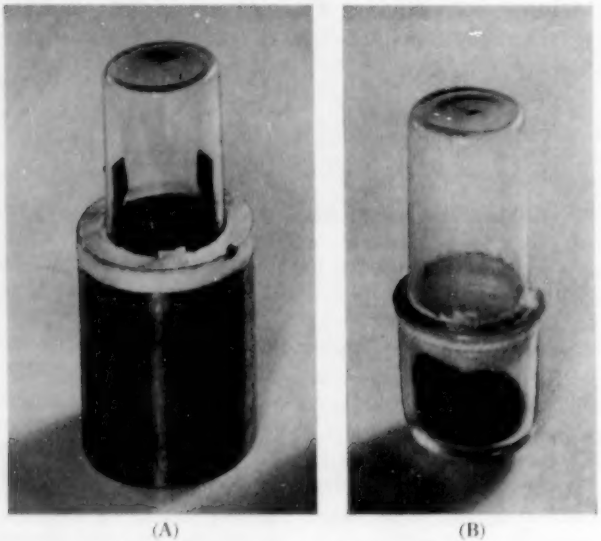
MATERIALS AND METHODS

The apparatus designed by us to feed fleas through an animal skin membrane consisted of two glass containers. The upper half, a glass shell vial, measured 2¼ inches in height with an outer diameter of 1¼ inches. The animal skin membrane, freshly prepared, was used without washing, freezing or subjection to defattening or depilatory substances. Blood containing plague organisms in 2.0 to 3.0 ml aliquot portions was placed into the vial and the membrane, with fur side outward, was stretched across the open end of the vial. The cover of a balsam bottle, measuring 1½ inches in height with an inner diameter of 1⅜ inches, was used as the lower half of the apparatus. Fine sand was poured into this container to a depth of approximately ¾ inch. When fleas were placed on the sand, the upper glass vial with membrane fur side downward was quickly inserted into the lower half (fig. 1A). An alternative apparatus used was essentially the same as the former except that the glass vial, with membrane attached, was held in place by a threaded steel collar which screwed snugly into a lower steel chamber (fig. 1B).

Adult fleas used in these experiments were either removed from the laboratory colony and starved for 3 to 7 days or were reared directly from larvae and the adults of known age afforded no opportunity to feed on an animal prior to use. When fleas in lots of 25 were placed in each feeding apparatus, the apparatus was immediately placed in a large desiccator jar. One hundred ml of 5% KOH solution which, placed within the jar, provided approximately 65% relative humidity. The top of the desiccator jar was tightly sealed and the fleas were allowed to feed at 25 C ±0.5 C.

In previous experiments, consistently negative results were obtained when fleas were given the opportunity to feed through a membrane with the blood on the underside of the membrane because of a micro-thin air layer which probably



(A) (B)

FIG. 1.—Flea feeding apparatus.

formed between the membrane and the fluid. Even when a very thin tissue-like membrane derived from the ear of a rabbit was used as a membrane, the fleas were unable to obtain a blood meal. Likewise, negative results were obtained with chick membrane (1 week old), parchment paper and with adult skins of *Mus musculus* (albino) and *Microtus montebelli*. However, when the position of fleas and blood was reversed, the fleas apparently encountered little difficulty in feeding even through the relative thick skin of *M. musculus* (albino). The best results obtained, however, in terms of percent feeding, occurred when adult *X. cheopis* fleas were allowed to feed overnight through a *M. musculus* (albino) skin membrance in contact with defibrinated rabbit blood. Sixteen and eight-tenths percent to 76% successful feedings were obtained per experiment or an average of 40.8% feedings, the figures representing percentages for males and females combined (table 1). It was noted that in all feeding experiments the males were more reluctant to feed than were the females. After feeding for 24 hours, the engorged males and females were separated, placed in test tubes containing fine wood shavings at the bottom and kept at 21 to 24 C until needed. Each flea to be dissected for culture was placed in a well of a sterile cardiolipin agglutination plate to which was added a small amount of sterile saline. Appendages and head were aseptically removed with sharpened surgeon's needles and gastrointestinal organs teased out gently into the saline solution. The proventriculus and stomach were then removed, washed in three changes of saline solution and macerated in a diluent of 1% peptone water.

RESULTS

Transmission attempts.—Specimens of *X. cheopis* used in these experiments received their infectious blood meal though the feeding apparatus previously described. Avirulent plague strain A1122, previously cultured at 30 C for

TABLE 1.—*Feeding records of* X. cheopis *after exposure for 24 hours to adult skin of* M. musculus (*albino*) *and to fresh defibrinated rabbit blood in the feeding apparatus.*

Batch no.	Number fed* / Number exposed		Percentage of fleas fed
1	9/25		36.0
2	14/25		56.0
3	10/25		40.0
4	37/93		39.8
5	35/100		35.0
6	16/95		16.8
7	28/75		37.3
8	98/210		46.7
9	38/50		76.0
Totals	285/698	(Average)	40.8

* Combined total of male and female fleas. Fleas starved 72 to 168 hours prior to feeding. Feeding temperature 25 C ± 0.5 C and relative humidity approximately 65%.

24 hours on blood agar slants and removed by washing with defibrinated rabbit blood was used as the infectious material. All the fleas were removed from the feeding apparatus after 24 hours and each flea examined microscopically for blood engorgement. The engorged specimens were segregated and kept at 21 to 24 C for 72 hours. At this time, the presumably infected fleas were allowed to feed en masse on a host mouse placed in a confinement cage within a glass battery jar. Fleas were periodically removed from the host animal and dissected for culture to determine the continued presence of plague bacilli. Each moribund host mouse was sacrificed, or upon death, autopsied, and heart and spleen cultured. A normal mouse was then placed in the cage with the fleas and the procedure repeated as long as any fleas remained alive. All transmission attempts were negative with infected *X. cheopis* from both Kobe, Japan, and California.

Blockage of digestive tract of X. cheopis.—Fleas were readily infected in the feeding apparatus through either chick (1 week old) or *M. musculus* (albino) skin membranes in contact with defibrinated rabbit blood concentration of 587×10^6 to 6382×10^6 plague organisms per ml. The use of heparin as an anticoagulating agent was avoided when it was observed that on occasions, the small volume of heparinized blood containing plague organisms clotted overnight at 25 C, thus preventing fleas from obtaining a blood meal. In one experiment, the fleas were infected through the feeding apparatus containing 720×10^6 organisms per ml. Even with this high concentration of organisms, viable plate counts were negative in the case of some of the males dissected for culture after the 30 minute and 24 hour feeding periods. After a 30-minute feeding opportunity the numbers of *Past. pestis* ingested by the males varied from 0 to 240 organisms, whereas in the females the counts varied from as low as 90 to as high as 75,000 organisms, the optimal ingestion ratio between males and females being approximately 1:300. Taking into consideration the maximum initial ingestion of plague bacilli by both sexes, there was a 100-fold increase in the females 48 hours after feeding. Although no data are available for males of the species after 48 hours, the concentration of viable organisms retained by the females in the proventriculus and stomach revealed a definite decrease 72 hours after feeding. Quan et al (1954) reported blockage of the proventriculus occurring as early as the 2nd day following an infectious meal. Our studies revealed the formation of proventricular masses with associated empty and constricted ventriculi in *X. cheopis* as early as the 3rd day and occasionally as late as the 9th day after an infectious meal. These proventricular masses were either broken immediately when the blocked fleas were given an opportunity to feed on a normal mouse or required 2 consecutive days of feeding of 5 minutes duration before breakage of the blocks occurred. In one experiment, 13 females were examined microscopically 7 days after an infectious meal. Microscopic observation revealed 5 fleas with dark proventriculi and constricted ventriculi. These 5 fleas, when fed individually on a mouse, broke their blocks immediately and were dissected for culture after the blood meal. Three isolates of *Past. pestis* were obtained. In one instance a female with a dark proventriculus and constricted ventriculus was encountered 3 days following an infectious meal. This female, when allowed to feed subsequently for 6 consecutive days, was still unable to break the block. No transmission occurred with this flea specimen.

DISCUSSION

Our investigations partially confirm Kartman's report that male *X. cheopis* do not feed as well as do females through membrane feeding techniques. Concerning the age of various rodent hosts whose skins were used in the feeding apparatus, Kartman reports that 49 of 128 male *X. cheopis* fed through juvenile *M. musculus* (albino) skins, whereas through adult skins of the same species of animal, not one male obtained a blood meal. This is at variance and contrasts sharply with our figures since our feeding experiments utilized skins removed from 20- to 25-g adult *M. musculus* (albino). Although in our feeding experiments more female than male fleas were used, positive feeding of male *X. cheopis* was obtained in all experiments conducted in spite of their apparent reluctance to feed. Since a high percentage of feeding did not always occur, particular emphasis was placed on the selection of fleas for experimental work. When fleas were to be selected from the rearing crocks, only healthy fleas amber in color and without digested blood in their midguts were removed and starved at 25 C for 3 to 7 days prior to use. Periodically the fleas were aspirated into a test tube from the feeding apparatus and microscopically checked for blood engorgement against a strong light to determine the feeding response. It was advantageous to use the lighter colored fleas in this work because of the ease in determining the presence of ingested blood in their digestive tracts. Many fleas were observed to feed immediately. However, all fleas were allowed to remain in the feeding apparatus to accommodate those individuals which were reluctant to feed at first in order to allow more time for them to become acclimated to their new environment before feeding. Kartman reported an average of 10.3% feedings of the total numbers of *X. cheopis* used when allowed to feed through adult *M. musculus* (albino) skins, whereas our average has been 40.8% feedings. Kartman obtained similar low feeding response with *X. cheopis* whether fresh or 5 day frozen blood of *Rattus norvegicus* was used in contact with adult *M. musculus* (albino) skins. On the other hand, Kartman obtained favorable feeding responses with skins of juvenile animals and with adult skins of other rodent species whose skins were thinner than that of *M. musculus* (albino). Although the thickness of the skin membrane may be a critical factor in feeding, our numerous attempts to feed fleas through skins of varied thicknesses from topside resulted in a negative feeding response possibly because of the formation of a thin air layer between the skin membrane and the blood. To minimize this air layer formation, fleas were allowed to feed from the underside of the membrane without subjection of skin membrane to washing, freezing, defattening or depilatory substances. Close cropping of hair from the skin membrane was not necessary. These in vitro studies suggest that basic problems relative to nutrition, physiology and disease vector efficiencies of different flea species obtained from widely separated geographic areas may be approached through the use of the feeding apparatus described in this report or through techniques similar in nature.

SUMMARY

A simplified apparatus is described for the experimental feeding and infection of fleas in vitro. A high percentage of fleas fed through an animal skin membrane in a desiccator jar maintained at approximately 65% relative

humidity and at 25 C ± 0.5 C. *X. cheopis* used in the studies fed readily through membranes prepared from the relatively thick skins of adult *M. musculus* (albino). The thickness of *M. musculus* (albino) skin apparently is not necessarily the limiting factor in the ability of *X. cheopis* to feed as reported by Kartman. The mode of infecting fleas through the feeding apparatus described is less complicated and lends itself to easier manipulation than does the apparatus described by Kartman.

REFERENCES

Kartman, L. 1954, Studies on *Pasteurella pestis* in fleas. I. An apparatus for the experimental feeding of fleas Exper. Parasitol. **3**: 525–537.

Quan, S. F., Kartman, L. and McManus, A. G. 1954, Studies on *Pasteurella pestis* in fleas: II. Experimental blocking of *Xenopsylla cheopis* with an avirulent strain of *P. pestis*. Science, **120**: 1101–1102.

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